Figure 5-7. Representative electron micrographs demonstrating effects of paclitaxel on cellular ultrastructure. Panel A shows the occasional microtubule (arrowhead) found in the cytoplasm of embryos treated with vehicle (Veh.; 0.1% DMSO) and panel B is a representation of the ultrastructure of the cytoplasm of embryos treated with vehicle. Panel B is a micrograph from an embryo treated with vehicle. Panels C-F are images of various structures induced by paclitaxel (Pacl.) including macrotubules (C), tubulin paracrystals (E), and blastomere fragments (D, F). Arrowheads point at microtubules (A), macrotubules (C), or paracrystals (E) and asterisks denote blastomere fragmentation (D, F). M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli, PVS = perivitelline space.

Figure 5-8. Representative electron micrographs illustrating distribution of mitochondria as affected by heat shock and cytoskeletal disruption agents. Shown are images from embryos cultured at 38.5 (A-F) or 43.0°C (G-H) in 0.1% ethanol (A), 0.1% DMSO (B), 1 μM Latrunculin B (C), 10 nM Rhizoxin (D, G), or 1 μM paclitaxel (E-F, H). Note the alignment of mitochondria and vesicles into columns oriented along the nucleus-periphery axis that occur when embryos are cultured in 1 μM paclitaxel (E-F) as compared to those with other treatments (A-D). Culture of embryos at 43.0°C induced mitochondrial swelling in the presence of paclitaxel (H) but not in the presence of the microtubule depolymerization agent rhizoxin (G). M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli.

CELLULAR, SUBCELLULAR, AND DEVELOPMENTAL RESPONSES OF TWO-CELL BOVINE EMBRYOS TO A PHYSIOLOGICALLY RELEVANT HEAT SHOCK

By

ROCIO MELISSA RIVERA

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I would like to dedicate this dissertation to three people who have been very influential in shaping my life as a person and scientist. First of all, my parents José D. Rivera and Zaida Varas who have showed me by example the importance of hard work, determination, thoroughness, and taking pride in ones work. I thank them for their love and support, and for giving me many opportunities to develop as a well-rounded person with a good balance between education, arts, and sports. The third person is my mentor and friend Dr. Peter J. Hansen. Dr. Hansen has been instrumental in my development as a scientist. His love for science and the pursuit of knowledge, cheerful disposition, and love for teaching and mentoring have been an incredible source of inspiration to me. These qualities have provided fertile soil for me to grow as a scientist and to fall in love again with science and the excitement of doing research. I will be forever be indebted to him for believing in me and for his patience, guidance, and encouragement.

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Abstract of Dissertation Presented to the Graduate School
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CELLULAR, SUBCELLULAR, AND DEVELOPMENTAL RESPONSES OF TWO-CELL BOVINE EMBRYOS TO A PHYSIOLOGICALLY RELEVANT HEAT SHOCK

By

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Exposure of preimplantation embryos to elevated temperatures blocks development. Temperatures used to demonstrate heat shock effects on development have been higher, however, than body temperatures typically experienced by heat-stressed cows. The goal of this dissertation was to determine the mechanism by which heat shock in the physiological range blocks development.

Initial findings demonstrated that development was disrupted by a short-term heat shock of 41.0°C or by continuous exposure to a 24-h pattern of temperatures similar to those experienced by heat-stressed cows. Ultrastructural changes induced in embryos by exposure to 41.0 or 43.0°C for 6 h were examined to determine mechanisms for inhibition of development. Heat shock of 41.0°C caused movement of organelles towards the center of the blastomere and an increase in the percent of mitochondria

exhibiting a swollen morphology. Exposure to 43.0°C (a non-physiological temperature) caused more widespread ultrastructural damage such as chromatin precipitation.

Experiments evaluated consequences of mitochondrial disruption caused by heat shock. Exposure to 41.0°C appears not to have an immediate effect on oxidative phosphorylation because heat shock did not immediately alter oxygen consumption or ATP content. It was observed that heat shock at the two-cell stage first caused a block in development at the eight-cell stage. Thus, damage to mitochondria or other structures at the two cell-stage first become critical to survival as the embryo enters the 8-16 cell stage.

An experiment was designed to test whether the heat shock-induced movement of organelles is a result of cytoskeletal rearrangement. At 41.0°C, movement of organelles from the periphery of the cell was blocked by rhizoxin, an agent causing microtubule depolymerization, and latrunculin B, a microfilament depolymerizer. Thus, disruption of both microfilaments and microtubules is involved in organellar movement. Only rhizoxin blocked organellar movement at 43.0°C, suggesting that microtubule damage at 43.0°C causes redistribution of organelles even when microfilaments are depolymerized. Microtubules are also involved in mitochondrial swelling because rhizoxin blocked effects of heat shock on mitochondrial swelling.

In conclusion, physiologically-relevant heat shock disrupts development of two-cell embryos and this action is mediated, at least in part, by disruptions of the mitochondria and cytoskeleton. Heat shock does not cause an immediate block to development in the two-cell embryo but prevents development past the eight-cell stage.

LIST OF ABBREVIATIONS

^{3}H – Tritium
^{35}S – Sulfur-35
3T3 cells – mouse, Swiss albino, fibroblast
ADP – Adenodisne Diphosphate
AIF – Apoptosis-Inducing Factor
Apaf-1 – Apoptosis activating Factor 1
ATP – Adenosine Triphosphate
Bim – Bcl-2-Interacting Mediator of cell death
BSA – Bovine Serum Albumin
BSO – Buthionine Sulfoximine
Ca^{2+}- Calcium
$CaCl_2$ – Calcium Chloride
Cl^- - Chloride
CHO – Chinese Hamster Ovary Cells
CO_2 – Carbon dioxide
d – day
DMSO – Dimethyl Sulphoxide
DNA – Deoxyribonucleic Acid
DNTB – 5,5-dithiobis(2-nitrobenzoic acid)
EDTA – Ethylenediaminetetraacetic Acid
EFAF BSA – Essentially Fatty Acid Free Bovine Serum Albumin
eIF-4F – Eukaryotic Initiation Factor-4F
ER – Endoplasmic Reticulum
FBS – Fetal Bovine Serum
FSH – Follicle Stimulating Hormone
G1 – Gap 1
G2 – Gap 2
GnRH – Gonadotrophin Releasing Hormone
GSH – Glutathione
h – hour
H^+ - Hydrogen
HCO_3^- - Bicarbonate
HCT116 cells – Human Colon Carcinoma cells
HeLa – human, Black, cervix, carcinoma, epitheloid
HEPES – Hydroxyethylpiperazine Ethanesulfonate
HL60 cells – Human, Caucasian, peripheral blood, leukemia
hpi – hours post-insemination
HSBP – Heat Shock Binding Protein
HSE – Heat Shock Element

HSF – Heat Shock Factor
Hsps – Heat shock proteins
IgG_1 – Immunoglobulin 1
K^+ - Potassium
KCl – Potassium Chloride
KCN – Potassium Cyanide
KH_2PO_4 – Potassium Phosphate Monobasic
KOH – Potassium Hydroxide
KRB – Krebs' Ringer Buffer
KSOM – Potassium Simplex Optimized Medium
KSOM BE1 – Potassium Simplex Optimized Medium Bovine Embryo 1
KSOM BE2 – Potassium Simplex Optimized Medium Bovine Embryo 2
M – Mitosis
$MgSO_4$ – Magnesium sulfate
MPT – Mitochondrial Permeability Transition
mRNA – messenger Ribonucleic Acid
Na^+ - Sodium
NaCl – Sodium Chloride
NADPH - Nicotinamide-Adenine Dinucleotide Phosphate, reduced
$NaPO_4$ – Sodium Phosphate
NRK cells – rat, Osborn-Mendel, kidney, fibroblast
O_2 - Oxygen
OCC – Oocyte Cumulus Complex
PBS – Phosphate Buffered Saline
PBS+PVP – Phosphate Buffered Saline + Polyvinyl Pyrrolidone
PBS+S – Phosphate Buffered Saline + Serum
PARP – poly(ADP-ribose)
$PGF_{2\alpha}$ - Prostaglandin F 2-alpha
PROC GLM – General Linear Model Procedure
PTP – Permeability Transition Pore
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
S – DNA replication phase
SAS – Statistical Analysis System
SEM – Standard Error of the Mean
SIS – Soft Imaging System
TALP – Tyrodes Albumin Lactate Pyruvate
TCM-199 – Tissue Culture Medium-199
V-79 – hamster, Chinese, lung, fibroblast
VDAC – Voltage Dependent Anion Channel
z-DEVD-fmk – N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone

PREFACE

Heat stress can reduce fertility in cattle (Dunlap and Vincent, 1971; Turner, 1982; Ealy *et al.,* 1993; Ryan *et al.,* 1993) and other species (Shah, 1956; Alliston and Ulberg, 1961; Tompkins *et al.,* 1967). Many methods based on reducing the magnitude of heat stress have been applied under farm conditions to ameliorate the hyperthermia experienced by the animal as a result of heat stress. Some of these include the addition of cooling systems to the barns, such as sprinklers, fans, and high-pressure foggers (Bray and Buclkin, 1997). However, fertility in the summer months, determined as 90 day non-return rate, is still less than 20% (Al-Katanani et al., 1999). Further approaches to improve fertility in dairy cattle may be possible as more is learned about the physiological mechanisms by which heat stress reduces fertility. It is known that the reduction in fertility observed as a result of heat stress is multifactorial in nature. For example, heat stress affects endocrine secretion of reproductive hormones (Gwazdauskas et al., 1973; Roman-Ponce et al., 1981; Badinga et al., 1993; Trout et al., 1998), follicular development (Badinga et al., 1993; Wolfenson et al., 1995), blood flow to the reproductive tract (Roman-Ponce et al., 1978), and oocyte competence (Al-Katanani et al., 1999). Several studies have also shown that the early mammalian embryo is very susceptible to elevated temperatures (Dutt, 1963; Elliot et al., 1968; Elliot and Ulberg, 1971; Ealy et al., 1993; Edwards and Hansen, 1997; Krininger et al., 2002). Taken

together, these observations suggest that the mechanisms of decreased fertility in the summer could be the result of disruption of embryonic development due to hyperthermia.

Susceptibility to hyperthermia experienced by early embryos is reduced as they advance through development (Ealy et al., 1993; Edwards and Hansen, 1997). For example, Ealy and coworkers (1993) showed that embryonic loss was greatest in cows that were heat-stressed on the first day after insemination when compared to cows heat-stressed on Days 3 or 5 after insemination (Day 0 = day of estrus). In addition, Edwards and Hansen (1997) demonstrated that two-cell bovine embryos produced in vitro were less tolerant to heat shock than in vitro-produced compact morulae (corresponding to Day 1 and Day 5 post-insemination, respectively). These data imply that it is during the first few cleavage divisions when embryos are most susceptible to the deleterious effects of elevated temperatures. Limited information has been gathered as to the mechanisms by which heat shock affects the early bovine embryo.

The goal of this dissertation is to evaluate whether heat shock in the physiologic range can disrupt embryonic development and to determine the basis for this effect at the cellular and sub-cellular levels. Demonstrating effects of physiologically-relevant heat shock on embryonic development would lend credence to the idea that infertility caused by exposure of cows to heat stress is, at least in part, a result of direct effects of maternal hyperthermia on embryonic development. Identifying the mechanisms by which heat shock causes these effects may lead to new strategies for improving fertility in the heat-stressed cow.

CHAPTER 1
REVIEW OF THE LITERATURE

Maternal Hyperthermia and Embryonic Loss – an Historical Account

It has long been recognized that elevated temperatures adversely affects fertility of females. As mentioned in the preface, the temporary infertility caused by heat stress is a multifactorial problem that involves alterations in several physiological systems including endocrine secretion of reproductive hormones (Roman-Ponce et al., 1981; Badinga et al., 1993), follicular development (Badinga et al., 1993; Wolfenson et al., 1995) blood flow to the reproductive tract (Roman-Ponce et al., 1978) and oocyte competence (Al-Katanani et al., 1999). To this mix of pathologies should be included inhibition of development and death of the preimplantation embryo caused by exposure to elevated temperatures. Indeed, the early preimplantation embryo is more sensitive to elevated temperature than most cells: temperatures as low as 39.0-41.0°C can inhibit embryonic development (Gwazdauskas et al., 1992; Dutt, 1963; Ealy et al., 1992). This section will use a historical approach to describe the experimentation that has led to the concept that elevated temperature is a key disruptor of embryonic development. Subsequent sections will develop concepts about how elevated temperature disrupts cells in general and preimplantation embryos in particular.

Concepts Derived From In Vivo Studies

Early research on the effects of heat stress on fertility in females led to an enhanced understanding of the role of this environmental stressor in the establishment and maintenance of pregnancy. Among the critical concepts demonstrated were the ideas that 1) heat stress decreases embryonic survival, 2) the effects of heat stress decline as pregnancy advances, and 3) actions of heat stress involve disruption to both the embryo and to the reproductive tract environment in which it resides.

Heat Stress Decreases Embryonic Survival

One of the first reports that fertility can be reduced by elevated temperatures dates back to a paper from Cordelia Ogle (1933) in mice. In that study, mice were caged under controlled environments at 16-20°C, 21-27°C, or 31-33°C. It was noticed that the females raised at 31-33°C had the lowest litter sizes.

From the early 1960's to the mid 1970's there was an explosion of information on effects of heat stress on early pregnancy in sheep (Alliston et al., 1961; Alliston and Ulberg, 1961; Dutt, 1963), mice (Aldred et al., 1961; Elliot et al., 1968; Elliot and Ulberg, 1971; Bellvé, 1972), pigs (Warnick et al., 1965; Tompkins et al., 1967; Edwards et al., 1968; Omtvedt et al., 1971; Wildt et al., 1975), and cows (Dunlap and Vincent, 1971). In all of these species, with the exception of the pig , clear evidence was provided that exposure of females to heat stress early in pregnancy leads to embryonic death.

In the sheep for example, lambing rate was significantly decreased for ewes that were placed in rooms maintained at 33°C for 24 h after mating (Dutt, 1963). That the decrease in lambing rate was due to embryonic loss was shown in another study in which ewes were maintained in a room at high temperature (33°C) for 8 days (from five days

from the expected time of estrus until three days after mating) and embryos recovered at Day 6 to 7 after mating. The majority of the embryos in the heat-stressed females were classified as abnormal and had halted development at the 32-cell stage (Alliston et al., 1961).

In one study, Elliot et al. (1968) evaluated the effects of exposure of females to 24-h of heat stress (34°C) beginning at ~16 h after mating (1600 h on the day that a copulatory plug was observed) on subsequent embryonic development and pregnancy outcome. A portion of the females were sacrificed ~50 h after mating and the rest on Day 10 of gestation. Embryos collected at ~50 h after mating were classified and cultured for three days. At the time of collection, there were more two-cell embryos in the heat-stressed females than in controls (22% vs. 3%) and a corresponding reduction in embryos at more advanced stages (34 vs. 51% eight cell embryos for heat stressed vs. control). None of the two-cell embryos continued development after culture. For embryos collected at later stages, fewer became blastocysts after culture when retrieved from heat-stressed females (45 vs. 62% for heat-stressed vs. control, respectively). On Day 10, there was a 21% decrease in the number of implantation sites in the heat-stressed group. These results confirm the idea that heat stress early in development can reduce embryonic development and suggests that in the mouse, at least, one major block to development occurs at the two-cell stage.

In 1971, Elliot and Ulberg reported a study that characterized effects of maternal heat stress during the first cleavage division on subsequent embryonic development. Females were assigned to be heat stressed (33°C) for a period of 24 h beginning approximately 16 h post-mating or remain at control temperature (21°C). Embryos

collected from heat-stressed females had a reduced number of cells when collected at ~60 h post-mating when compared to embryos collected from control females. In addition, fewer successful pregnancies resulted when females were heat-stressed (47% vs. 81% for heat-stressed and control females, respectively). Figure 1-1 shows the percent of embryos found at each stage of development at ~60 h post-mating. As the graph demonstrates, 80% of the embryos from heat-stressed females were halted in development before the eight-cell stage while only 23% of embryos from control females were less than eight cells in number.

Similar to results of Elliot and Ulberg (1971), Bellvé (1972) showed that heat stress of female mice at 34.5°C for 24 h beginning approximately 16 h after mating caused extensive retardation of embryonic development (Figure 1-2). As compared to embryos from controls, there was an increased proportion of two-, three-, and four-cell embryos and a decreased proportion of eight-cell embryos recovered at ~60 h post-mating.

The first study documenting an effect of maternal heat stress on embryonic survival in cattle was by Dunlap and Vincent (1971) who studied the influence of post-breeding thermal stress on conception rates in beef cattle. In this experiment, beef heifers were exposed to 32.2°C for 72 h following breeding. None of the heat-stressed heifers calved while 48% of the control group maintained at 21.1°C calved. The next study demonstrating effects of matemal heat stress on embryonic survival was reported by Putney et al. (1988). In this study, superovulated heifers were used as a model to determine the effects of heat stress during the first seven days of pregnancy on embryonic

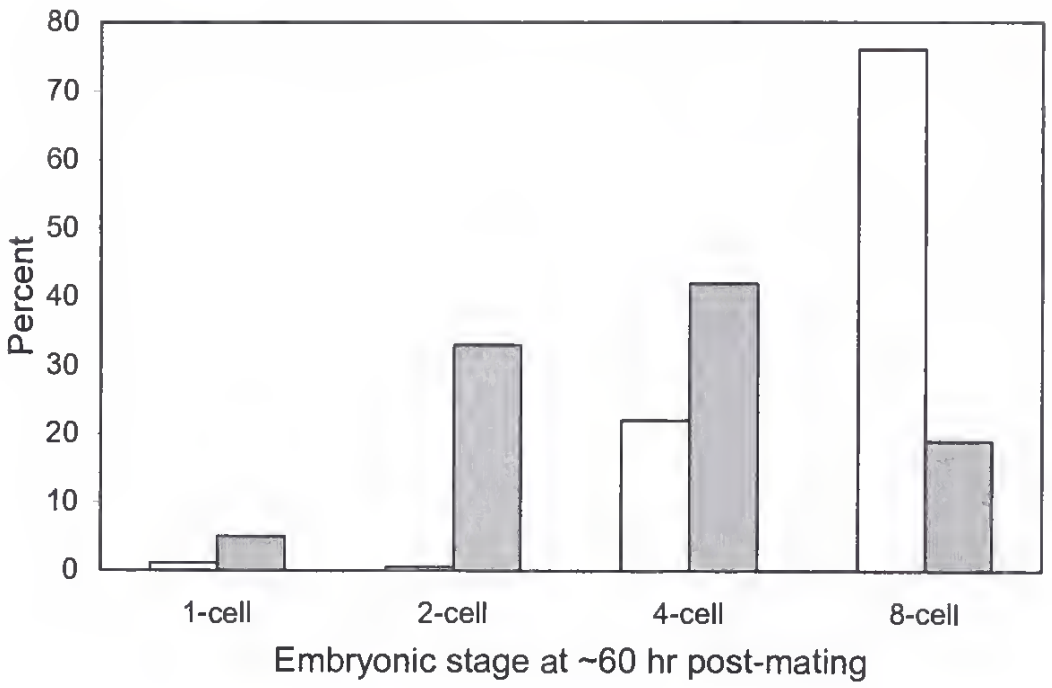


Figure 1-1. Summary of the effects of heat stress on percent of embryos at the 1-, 2-, 4-, and 8-cell stage of development at ~60 h post-insemination. Females were heat stressed (33°C) or left as control (21°C) for a period of 24 h beginning approximately 16 h post-mating. Data presented in this graph represent the average of three individual experiments performed by Elliot and Ulberg (1971). Open bars represent embryos from control females (n = 49) and grey bars represent embryos from heat-stressed females (n = 51).

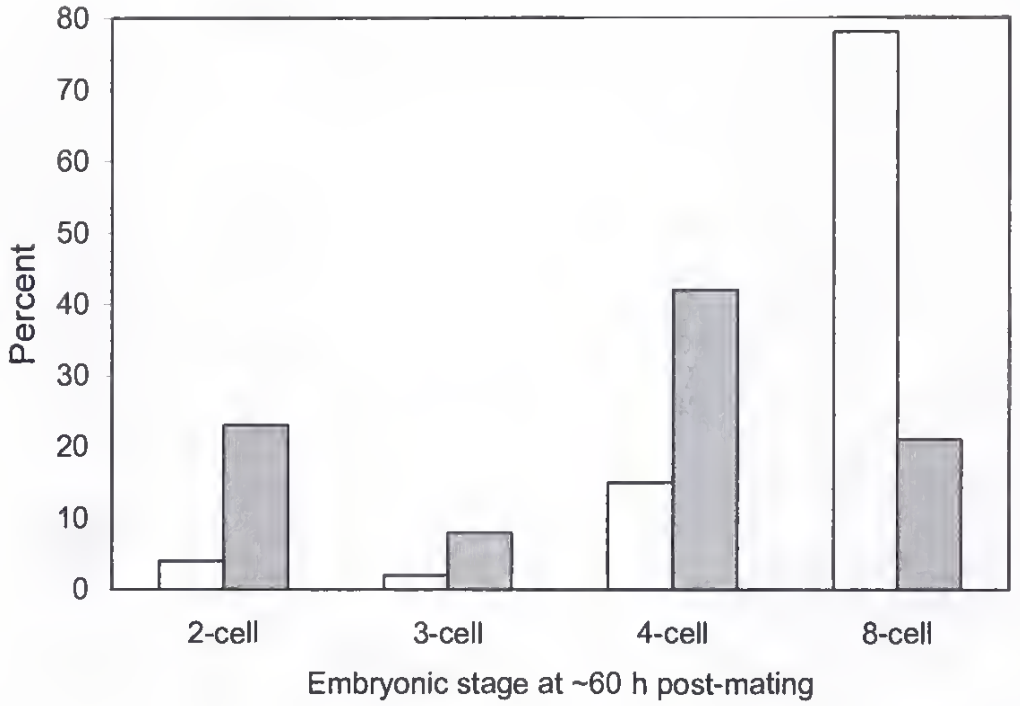


Figure 1-2. Effects of a 24 h heat stress beginning at approximately 16 h post-insemination on percent of embryos at the 1-, 2-, 4-, and 8-cell stage. Embryos were recovered from females at ~60 h post-insemination. Data presented in this graph are taken from Bellvé (1972). Open bars represent embryos from control females (n = 96) and grey bars represent embryos from heat-stressed females (n = 96). Note that numbers are approximations as they were estimated from a graph in the original paper.

development. Heifers were superovulated and artificially inseminated. Beginning at 30 h after estrus, heifers were either maintained at 20.0°C or exposed to a heat stress for 6 days that consisted of daily exposure to 30.0°C for 16 h and 42.0°C for 8 h. Embryos were recovered at Day 7 after insemination. Heat stress reduced the percent of embryos having good to excellent quality from 58% in the control group to 45% in the heat-stressed group. Furthermore, the percent of retarded and abnormal embryos increased as a function of increased rectal temperatures.

Collective reports regarding effects of heat stress in the pig are difficult to interpret because of contradictory data. In addition, experiments with this species are more varied as to duration of heat stress as well as the time during early pregnancy when heat stress was applied. In general, however, results generally point to a reduction in survival of preimplantation embryos as a result of maternal hyperthermia. Nevertheless, the pig seems much more resistant to heat-stress associated embryonic mortality than some other species.

In an early experiment, Warnick et al. (1965) found that exposure to heat stress of 32.3°C for the first three days after mating did not affect the number of live embryos on Day 25 of pregnancy. More prolonged heat stress, for 25 days, did reduce the number of live embryos observed at Day 25 of pregnancy. The study of Warnick et al. (1965) is illustrative of the severe heat stress required to disrupt embryonic development in the pig. Others have observed similar results. Tompkins et al. (1967) failed to find an effect of a 24 h heat stress of 35.0°C given on Day 1, 3, or 5 after breeding on embryonic survival at Day 35 of pregnancy. Although not statistically significant, that data showed a 30% reduction in viable embryos when heat stress was applied on Day 1. When the heat stress

temperature was increased to 36.7°C and the duration of heat stress extended to five days beginning Day 1 of gestation, there was only a slight reduction in embryonic death despite the death of four of nine heat-stressed sows. Edwards et al. (1968) observed that heat stress reduced the number of viable embryos on Day 33 post-breeding but the heat stress was exposure to 38.9°C for 17 h per day for the first 15 d post-breeding. In this study, too, some animals died as a result of heat stress. Likewise, Omtvedt et al. (1971) observed that a heat stress regimen of 17 h daily at 37.8°C for 8 days resulted in a 43% reduction in pregnancy rate on Day 33 post-breeding.

Even when heat stress reduces embryonic survival in pigs, not all animals experience embryonic death. Wildt et al. (1975) exposed pigs to 40.4°C for 2 h each day for 12 days beginning on Day 2 post-breeding. While overall heat stress increased embryo mortality from 35% to 63%, loss of embryos was not seen in all animals. Of the ten pigs exposed to heat stress, four experienced an average embryonic loss of 93% whereas the remaining animals had embryonic mortality similar to controls.

Taken together, it would appear that the pig is not a good general model for understanding early embryonic death in response to heat stress. Perhaps the early porcine embryo is not as susceptible to the effects of elevated temperature as embryos of other species. Another possibility is that the pig has better ability for thermoregulation as compared to some other species. In the work of Warnick et al. (1965), Omtvedt et al. (1971) and Wildt et al. (1975), pigs tended to adapt to heat stress so that body temperature became progressively closer to controls as the experiment progressed.

Changes in Sensitivity to Heat Stress as Pregnancy Advances

The first report to indicate that the maternal system becomes more tolerant to the effects of heat stress as pregnancy progresses was provided by Fernandez-Cano (1958) using the rat as a model. A change in maternal body temperature from 37 to 39°C on Day 1 and 2 of pregnancy increased the number of degenerated embryos from 2.5% in control rats to 65% in heat stressed rats. In contrast, hyperthermia during Days 3-4 or 6-7 of pregnancy reduced the percent of degenerated embryos to 30 and 15%, respectively.

Similar results were obtained in the mouse by Aldred et al. (1961). In this study, mice were subjected to 40°C for 1, 3, or 5 h on Day 1 following mating, for 5 h on Day 2 after mating, or for 5 h on Day 1 and 2. The number of viable and reabsorbed embryos was determined as a percent of the number of corpora lutea following autopsy on Day 17. Their data show that embryonic loss due to heat stress on Day 1 increased as the duration of heat stress increased (embryonic loss – control = 14%, 1 h = 15%, 3 h = 17%, 5 h = 19%). Also, there were less severe effects of 5 h of heat stress on Day 2 (embryonic loss = 10%) than when females were stressed on Day 1 and 2 for 5 h each (embryonic loss = 33%).

Results in accordance with findings in rodents were obtained in the sheep by Dutt (1963). Ewes were exposed to high temperatures from Day 0-1, 3, or 5 after breeding until Day 24 of pregnancy and then returned to control temperature until lambing. The proportion of ewes lambing was 85% for control ewes and 10, 35 and 40% for ewes heat-stressed beginning on Day 0, 3, and 5, respectively. When a subset of ewes were analyzed on Day 3 after mating, it was found that 38.5% of embryos in heat-stressed ewes were classified as abnormal versus only 3.7% of embryos from control ewes.

In the cow as well, the magnitude of the disruption in embryonic survival caused by heat stress decreases as pregnancy advances. Such a conclusion was derived from the work of Ealy et al. (1993), who exposed superovulated cows to a single day of heat stress at 1, 3, 5, or 7 d after insemination and then measured embryo survival at Day 8. To induce heat stress, cows were placed in an unshaded lot from 0800 h to 1500 h. The percent of embryos classified as live based on vital dye staining was 70% for control cows and 55, 68, 65, and 89% for cows heat-stressed on Days 1, 3, 5, or 7, respectively. The percent of embryos that were blastocysts was reduced from 49% in the control cows to 34% for cows heat-stressed at Day 1. Heat stress at other days had no effect on percent blastocyst (60, 57, and 64% for Day 3, 5, or 7, respectively).

Effects on the Reproductive Tract vs. Embryo

The reduction in embryonic survival caused by maternal heat stress administered after fertilization could be a consequence of direct actions on the embryo or indirect actions caused by alterations in the reproductive tract environment. Using the rabbit as a model, Shah (1956) was the first to conduct an experiment to study the embryo-uterine relationship during heat-induced pregnancy failure. It was concluded that effects of heat stress were mediated by changes in the uterine environment because embryos from heat-stressed females that were transferred to a non-stressed female could establish pregnancy.

A similar experiment was performed in ewes by Alliston and Ulberg (1961). Three experimental groups were used – ewes raised in a control environment (housed at 21°C) that received an embryo from a female in a control environment, ewes raised in a control environment that received an embryo from a female exposed to heat stress (housing at 32°C), and ewes raised in a heat-stress environment (housed at 32°C). Heat

stress was performed from 5 d before estrus until the time of transfer. Therefore, effects on the oocyte and embryo were possible. Embryo transfers were performed around 72 h post-insemination (approximately at the eight-cell stage) and pregnancy was determined on Day 25-30 post-mating. Pregnancy rates for the three groups were 56% (control embryos in control ewes), 10% (heat-stressed embryos in control ewes) and 24% (control embryos in heat-stressed ewes). Thus, heat stress appears to affect both the embryo and reproductive tract with the former effects being more severe.

Summary of Experiments Concerning Effects of Maternal Hyperthermia on the Early Preimplantation Embryo

The experiments described in this section provide abundant evidence that the processes leading to embryonic survival during early pregnancy can be disrupted by exposure of pregnant females to heat stress. Moreover, the female is particularly sensitive to effects of heat stress in the first few days of pregnancy. Experiments with reciprocal embryo transfer lead to the conclusion that heat stress can compromise embryonic survival by affecting the embryo directly and by altering the reproductive tract environment. One problem with the reciprocal transfer approach is that a reduction in pregnancy rate in control animals receiving embryos from heat-stressed females could be result of direct damage to embryo or effects on the reproductive tract to cause permanent disruption of the embryo that cannot be corrected by transfer into the reproductive tract of a non-stressed female. Further evidence that the preimplantation embryo is susceptible to heat stress requires use of cultured embryos to study effects of heat shock on the embryo independent of alterations in the reproductive tract.

Concepts Derived From In Vitro Studies

Rather than examine the effects of heat stress (i.e., the sum of the environmental conditions that determine an animal's heat balance and which drive it into hyperthermia), experiments with cultured embryos have focused on heat shock (a chronic or acute elevation in temperature applied to a cell, organ, tissue, or organism). In vivo, mammalian or avian cells become exposed to heat shock when body temperature rises because of fever or heat stress. Heat shock can be induced in cultured cells and tissues by exposure to culture conditions involving elevated temperature. Such experiments with cultured embryos are necessary for two reasons; 1) to determine direct effects of elevated temperature on embryonic development and 2) to determine the mechanisms involved in the disruption of embryonic function caused by heat stress.

Heat shock directly applied to the embryo has been reported to decrease development in many species including mouse (Gwazdauskas et al, 1992; Aréchiga et al., 1995; Edwards et al., 1997; Aréchiga and Hansen, 1998), cow (Ealy et al., 1995; Edwards and Hansen, 1996; Ju et al., 1999; Al-Katanani and Hansen, 2002; Block et al., 2002; Krininger et al., 2002; Paula-Lopes and Hansen, 2002a; Paula-Lopes et al., 2003a,b), rabbit (Alliston et al, 1965), and pig embryos (Kojima et al., 1996). Studies both in vivo (Fernandez-Cano, 1958; Alliston and Ulberg, 1961; Alliston et al., 1961; Dutt, 1963; Elliot et al., 1968; Elliot and Ulberg, 1971; Dunlap and Vincent, 1971; Bellvé, 1972; Putney et al.,1988; Ealy et al., 1993) and in vitro (Gwazdauskas et al, 1992; Ealy et al., 1995; Edwards and Hansen, 1997; Aréchiga and Hansen, 1998; Al-Katanani and Hansen, 2002; Block et al., 2002; Krininger et al., 2002; Paula-Lopes and Hansen, 2002a; Paula-

Lopes et al., 2003a,b) show that temperatures as low as 39.0-41.0°C render the embryo unable to continue development.

Preimplantation embryos are susceptible to elevated temperature from the one-cell stage though the blastocyst stage of development (Ealy and Hansen, 1994; Ealy et al., 1995; Edwards et al., 1997; Aréchiga and Hansen, 1998; Ju et al., 1999; Al-Katanani and Hansen, 2002; Block et al., 2002; Krininger et al., 2002; Paula-Lopes et al., 2003b).

The previous statement should not be interpreted to mean that all stages of embryonic development are equally sensitive to heat shock. There is evidence in several species that heat shock effects are reduced as development advances. The most severe effects of heat on developmental competence of embryos usually occur before the eight-cell stage and more specifically during the first two cleavage divisions (Gwazdauskas et al., 1992; Edwards et al., 1997; Krininger et al., 2002). From the eight-cell stage onwards, preimplantation mammalian embryos gain the ability to withstand the deleterious effects of heat (Ealy et al., 1995; Edwards and Hansen, 1997; Aréchiga and Hansen, 1998; Ju et al., 1999; Krininger et al., 2002). For example, Edwards and Hansen (1997) showed that a heat shock of 41.0°C for 12 h obliterated development of two-cell bovine embryos to the blastocyst stage while only slightly reducing development of four- to eight-cell embryos and having no effect on compacted morulae. Similarly, Krininger et al. (2002) showed that two-cell bovine embryos were severely affected by an elevation in temperature than embryos at Day 5 post insemination that were 16-cells or greater. Moreover, in the mouse (Aréchiga and Hansen, 1998), a heat shock of 41.0°C for 1 h at the two-cell stage reduced the percent of embryos developing to blastocyst from 98% in control to 75%. On the other hand, when a similar heat shock was applied at the morula

stage there was no difference in the percent of embryos becoming blastocysts (95 and 90%, for control and heat shocked embryos, respectively). In addition, Muller et al. (1985) observed differences in the sensitivity to heat shock between one-cell and blastocyst stage mouse embryos with the former being extremely sensitive and the later being resistant. Taken together, these data would suggest that some of the differences observed in pregnancy responses when heat stress is applied on different days after conception (Fernandez-Cano, 1958; Dutt, 1963; Ealy et al., 1993) reflect, at least in part, changes in embryonic responses to heat shock.

In addition to the developmental differences in resistance to heat shock, several studies have demonstrated that there is a genetic component controlling embryonic responses to heat shock. For instance, Paula-Lopes et al. (2003b) demonstrated that the deleterious effect of heat shock at Day 5 after insemination on blastocyst formation and the number of cells per embryo was less pronounced for Brahman embryos than embryos of the Holstein or Angus breeds. Similarly, Hernández-Cerón et al. (2003) found that embryos from the Romosinuano (a heat tolerant breed of *Bos taurus* genetic origin) and Brahman (heat tolerant breed of *Bos indicus* genetic origin) were more tolerant of heat shock applied to embryos ≥ 8-cells than embryos from the Angus breed (a heat sensitive breed of *Bos taurus* genetic origin). When heat shock was applied at the two to four cell stage, however, there were no differences in resistance to heat shock between Brahman and Holstein embryos (Krininger et al., 2003). Thus, genetic differences may require advanced development, perhaps in conjunction with embryonic genome activation. There is also evidence that genetic resistance to heat shock is inherited maternally (Block et al., 2002). In that study, heat shock ≥ 9-cell stage reduced the percent of embryos

formed from insemination of Holstein oocytes with Angus sperm developing to blastocyst from 56 to 30% while causing a slight increase in development for embryos formed from insemination of Brahman oocytes with Angus sperm (42 vs. 56% for control vs. heat-shocked, respectively). In contrast to this effect of breed of oocyte, there was no difference in resistance to heat shock for embryos derived from Holstein oocytes regardless of whether spermatozoa used for fertilization were from Brahman or Angus bulls. These results were interpreted to mean that either 1) genes determining thermotolerance are paternally imprinted or 2) are dependent upon a preformed factor in the oocyte.

A question that arises from all these studies is; what is the cellular mechanism whereby heat shock renders the preimplantation embryo unable to continue development? Limited information has been gathered on the direct effects of heat shock at the cellular level on embryos. Therefore, the approach will be taken to review the literature on the effects of heat shock on other cell types and, when possible, describe information collected thus far on the mammalian preimplantation embryo.

Cellular, Molecular, and Ultrastructural Effects of Heat Shock on Cells and Embryos

Cytoskeleton

One of the most noticeable effects of heat shock on cells are changes in cell shape. Several studies have demonstrated that, upon heat shock, one or many of several cell shape changes can occur, as for example, cell flattening, cell rounding, and membrane blebbing (Coss and Linnemans, 1996). These changes are a result of

cytoskeletal reorganization (Glass et al., 1985; Shyy et al., 1989; Coss and Linnemans, 1996; Wang et al., 1998).

The cytoskeleton is a cellular component whicb is common to the plasma membrane, cytoplasm, and nucleus. The cytoskeleton plays important roles in 1) regulation of cell structure (by supporting the cell membrane), 2) cell movement, 3) cytokinesis, 4) binding of cell components such as enzymes and messenger RNA, and 5) redistribution of cell organelles through the actions of molecular motors (Gallicano et al., 1994; Brown, 1999; Lodish et al., 2000; Kamal and Goldstein, 2000).

This dynamic and extensive intracellular network is composed of three types of fibers, namely, 1) microfilaments, 2) intermediate filaments, and 3) microtubules (Lodish, 2000). Microfilaments are composed of actin subunits, microtubules of tubulin subunits, and the intermediate filaments of cell-specific proteins (i.e. cytokeratins, vimentin, desmin, lamins) which are grouped into five classes (types I to V; Hermann and Aebi, 2000). Microfilaments and microtubules are highly dynamic systems (Coss and Linnemans, 1996) which show a steady-state equilibrium between the polymerized structures and large unpolymerized pool of subunits. It is for this reason that the organization of these networks can be quickly altered as a result of polymerization or depolymerization. Although dynamic changes occur in intermediate filaments also, the pool of unpolymerized subunits is small (Eriksson et al., 1992) and it has been speculated that phosphorylation and dephospborylation cycles control their dynamic properties.

Exposure of primary cultures of mouse epithelial cells to elevated temperatures resulted in change in the organization of keratin filaments and actin filaments while having no effect on microtubules (Shyy et al., 1989). Similar results were observed in 9L

cells where microtubules were only slightly affected by heat shock while cytoplasmic microfilaments and intermediate filaments collapsed (Wang et al., 1998). Rearrangement of microfilaments as a result of heat shock have been observed by Iida et al. (1986) who demonstrated that heat shock of 42.0-43.0°C causes disintegration of normal actin and induced formation of intranuclear actin paracrystals and actin rods. Detection of the intermediate filament protein vimentin revealed that heat shock of 45.0°C for 30 min caused the intermediate filaments to rearrange from an elaborate lattice work in the cytoplasm to a cage-like form around the nucleus (Cheng and Lai, 1994). Similar collapse of intermediate filaments as a result of heat shock have been observed in the chicken fibroblast (Collier et al., 1993) but not in NRK cells in which intermediate filaments are very resistant to elevated temperatures (Ohtsuka et al., 1993).

In contrast to the lack of effect on the microtubules in the previously cited works, heat shock can disrupt microtubules in Chinese hamster ovary (CHO) cells (Coss et al., 1996) and mouse 3T3 cells (Lin et al., 1982). Further, it has been documented that microtubules isolated from calf brain (body temperature of 38.5°C) will disassemble at 41.0°C and will denature at 43.0°C (Turi and Lu, 1981). From these results, it is obvious that the effects of heat shock on the different cytoskeletal components vary depending on cell type (for a review see Coss and Linnemans, 1996).

Organelles

Another major effect of heat shock in cells is the induced redistribution of organelles to the perinuclear region as well as changes to organelle morphology (Shyy et al., 1989; Cole and Armour, 1988; Welch and Suhan, 1985; Wang et al., 1998; Funk et al., 1999). These observations are very consistent among different cell types and appear

to be due to cytoskeletal rearrangement (Welch and Suhan, 1985; Coss and Linnemans, 1996; Wang et al., 1998). Using both electron microscopy and immunological methods, Welch and Suhan (1985) characterized a number of morphological changes occurring in rat embryo fibroblast cells after a heat shock of 42.0°C for 3 h. They demonstrated mitochondrial mobilization to the vicinity of the nucleus as a result of heat shock as well as a number of structural changes within the mitochondria. Specifically, the mitochondria appeared swollen, the cristae more prominent, and the intracristae spaces appeared enlarged. These authors suggested that this relocalization of mitochondria may be due to a collapse and aggregation of the intermediate filaments around the nucleus of heat-treated fibroblasts. Funk et al. (1999) demonstrated by means of electronic light microscopy, confocal laser scanning microscopy, and electron microscopy, that heat shock caused mitochondria to become swollen, to change from a rodlike shape into an annular-shaped organelle and to experience a reduction in motility. Similar results were observed by Wang et al. (1998) who demonstrated that in normal cells, mitochondria are mostly long and straight and evenly distributed in the cytoplasm. Upon heat shock, these filamentous mitochondria changed their morphology rapidly and became shortened and swollen and migrated towards the perinuclear region where they became mostly granular. After heat shock, the Golgi apparatus dissociated into numerous small vesicles. In addition, Welch and Suhan (1985) observed that the Golgi complex as well as the endoplasmic reticulum (ER) appeared to be fragmented and had formed vesicles which could be found throughout the perinuclear region.

Of particular interest is the mitochondrial swelling observed as a result of heat shock. Mitochondria are very important organelles as they produce the energy for the

cell in the form of ATP, are tightly integrated in cellular calcium signaling, serve as stress indicators for the cell, and play a crucial role in apoptosis as one of the central signaling systems for activation of execution caspases in programmed cell death (Duchen, 1999; Funk et al., 1999). As mentioned previously, heat shock has profound effects on this organelle. Funk et al. (1999) showed that after heat shock the mitochondrial membrane potential was depressed in most mitochondria as monitored with the fluorescent probe JC-1. The loss in membrane potential as a result of heat shock can have devastating results for the mitochondria and the cell as it could result in the release of apoptotic proteins from the intermembrane space. Hyperthermia has been noted to impair electron transport along the respiratory chain (Floridi et al., 1987) resulting in a decrease in the level of ATP and phosphorylation efficiency (Findly et al., 1983; Calderwood, 1987; Macouillard-Poulletier de Gannes et al., 1998). Since mitochondria undergo temperature-dependent uncoupling during increases in temperature (Floridi et al., 1987; Flanagan et al., 1998; Kourie, 1998), it is likely that heat-shocked cells would have a different rate of oxygen consumption than controls cells. In fact, Macouillard-Poulletier de Gannes et al. (2000) showed that the respiratory properties of microglial cells are compromised during and immediately after heat shock as measured by and immediate and strong drop in oxygen consumption. In addition, Calderwood et al. (1985) showed that ATP content of cells decreased as a result of heat shock.

Membranes

One of the effects of heat shock on cells is altered physiology of cellular membranes (Lepock, 1982; Gerner et al., 1980; Arancia et al., 1989; Bates et al., 1996; Coss and Linnemans, 1996). Changes as a result of heat shock include accumulation of

electron-dense particles, discontinuous plasma membranes, loss of microvilli, and lifting of the plasma membrane from the underlying cytoplasm (Bowler, 1987; Arancia et al., 1989). In addition, other modifications observed to occur during heat shock are changes in plasma membrane fluidity, changes in membrane potential, and the loss of activity of membrane-bound proteins (Coss and Linnemans, 1996). Changes in membrane potential have been observed in plasma membranes of hamster lymphocytes exposed to heat (Marcocci and Mondovi, 1990). Heat shock generally enhances membrane permeability and thus increases the flux of ions and small molecules (Gerner et al., 1980; Marcocci and Mondovi, 1990; Kuhl and Rensing, 2000) so that transmembrane gradients of ions and metabolites are disturbed (Marcocci and Mondovi, 1990). For example, exposure of cells to heat shock causes the plasma membrane to become leaky to polyamines (Gerner et al., 1980). In addition, heat shock of 43.0°C results in increases in intracellular calcium ions due to an influx into the cytoplasm from both internal stores and the extracellular medium (Itagaki et al., 1998). It has been suggested that the stress-induced disturbances of Ca^{2+}- and thiol homeostases (specifically glutathione: GSH) affect the cortical cytoskeletal network of isolated hepatocytes and, subsequently, the integrity of the plasma membrane, leading to bleb formation (Jewel et al., 1982).

Changes in temperature will disrupt both the lipid and the protein moieties that make up membranes. An increase in temperature will increase the molecular motion of lipids, due to an increase in kinetic energy, which results in an increase in fluidity (Lee and Chapman, 1987). In an unsaturated fatty acid requiring mutant of *Escherichia coli*, there is evidence for a correlation between membrane microviscosity (reciprocal of fluidity) and sensitivity to hyperthermia (Dennis and Yatvin, 1981). However, increasing

microviscosity of ascites and V79 cell membranes did not influence the response of cells to heating (Yatvin et al., 1983). In 1980, Gerner et al. proposed a model for thermal killing in which removal of cholesterol molecules (a molecule involved in cell membrane fluidity) via the action of polyamines (ubiquitously occurring polycations that have been demonstrated to sensitize cells to heat) makes the plasma membrane more fluid and leaky resulting in the inability of the cell to exert normal osmotic controls. Further, heat shock does not alter levels of the major phospholipids such as phophatidylcholine, phophatidylserine, and phosphatidylethanolamine (Bates et al., 1996).

There is also evidence to suggest that membrane proteins, possibly those involved in intracellular pH regulation and lipid-protein interactions, can be disrupted by heat shock (Coss and Linnemans, 1996). The regulation of intracellular pH is a vital aspect of cell homeostasis (Dale et al., 1998). Major intracellular processes are highly sensitive to intracellular pH including protein synthesis, cell metabolism, Ca^{2+} homeostasis, mitochondrial function, gene expression, cytoskeletal regulation, and cell death (Puceat, 1999; Dale et al., 1998). The ion transport mechanism is crucial in maintaining intracellular pH in all cell types. Interestingly enough is the fact that in monkey kidney Vero cells, heat shock has been shown to decrease the activity of the Na^+-independent HCO_3^-/Cl^- exchanger (Oehler et al., 1998). Further, heat shock has been demonstrated to result in acidification of cells (Oehler et al., 1998; Kiang and Tsokos, 1998).

Although little is known regarding effects of heat shock on membrane functions in preimplantation embryos, it is likely that cells of these organisms are less likely to adjust to disruptions in membrane function than many other cells. Observations by Baltz

et al. (1991) showed that none of the known intracellular pH regulatory mechanisms found in other mammalian cells (i.e. Na^+/H^+ antiport, Na^+/H^+ pumps, K^+/H^+ pumps) are active in two-cell stage mouse embryos during recovery from acidosis. They concluded that recovery from acid loads in the two-cell mouse embryo is a passive process following the electrochemical gradient across the plasma membrane (Baltz et al., 1991; Baltz, 1993). Recovery from alkaline loads in two-cell mouse embryos, however, involves the HCO_3^-/Cl^- exchanger (Baltz et al., 1991).

Nucleus, Nucleic Acids, Cell Cycle, and Mitosis

The nucleus contains thermolabile proteins that denature and aggregate as a result of exposure of hydrophobic domains during heat shock (Lepock et al., 2001). The aggregated protein in the nucleus can be observed by electron microcopy as regions of electron-dense material (Swanson et al., 1995). Further, Welch and Suhan (1985) showed that upon heat shock, fibrous containing, rod-like structures appeared within the nucleus. These inclusion bodies appeared to be packed with thin filaments. Using meromyosin decoration studies, these authors determined that the inclusion fibers contained actin. This study suggests that disruption of the nuclear matrix involves cytoskeletal rearrangement.

Upon heat shock, nuclear chromatin loses it's normal appearance (Swanson et al., 1995), nucleoli became less condensed and show signs of unraveling, and aggregation of denatured proteins at the nuclear matrix are observed (Welch and Suhan, 1985; (Hildebrandt et al., 2002). It is well established that DNA synthesis, as measured by incorporation of precursors such as [^{3}H]thymidine, is rapidly inhibited by heat shock (Marcocci and Mondovi, 1990; Warters and Roti Roti, 1982). Exposure of HeLa and

Chinese hamster V79 cells to heat shock results in the inhibition of replicon initiation and DNA replicative elongation and inhibition of DNA repair synthesis (Marcocci and Mondovi, 1990). Warters and Roti Roti (1982) have suggested that the inhibition of DNA synthesis is a result of heat-induced denaturation of replicative enzymes since DNA polymerase α and β are both inhibited by heat shock. Furthermore, heat shock reduces [^{3}H]uridine incorporation in HeLa cells (Burdon, 1987) indicating reduced transcription as a result of elevated temperatures.

In most cases, heat shock causes antiproliferative effects (Huang et al., 1999; Kuhl and Rensing, 2000). Differential effects of heat have been observed during the different phases of the cell cycle. The cell cycle is composed of four phases that proceed successively in most growing cells. These are three cycles that make up the interphase - G1 (the first gap - before DNA synthesis occurs), S (phase where DNA replication occurs) and G2 (the second gap - after DNA synthesis) – and the M phase (or mitosis) during which time occurs condensation of the duplicated chromosomes, their alignment at the metaphase plate, separation of the sister chromatids, and the segregation into two daughter cells (Lodish et al., 1995). The heat shock-induced rearrangement of the cytoskeleton, especially of the tubulin system interferes, with the normal distribution of the genetic material to the daughter cells and arrests the cell cycle (Huang et al., 1999). In a study using mouse oocytes, for example, (Fiorenza and Mangia, 1992), mild heat shock conditions during oocyte maturation disrupted the process of bivalent chromosome disjunction, blocking oocytes at the metaphase I stage. In addition, heat shock induced the appearance of abnormal chromosome morphology and number. Even though the authors did not look at effects of heat shock directly on the cytoskeleton, the unequal

chromosome segregation suggests cytoskeletal and especially microtubule damage. Heat shock of CHO cells also caused alterations to the centrosomes resulting in multipolar mitotic spindles, delay in prophase-metaphase, and formation of multinucleated cells which were non-clonogenic (Vidair et al., 1993). Heat shock of HeLa cells at 41.5°C resulted in asymmetric segregation of chromatid clusters and premature reformation of the nuclear membrane resulting in multiple micronuclei formation (Swanson et al., 1995).

In mammalian cells, heat shock leads to a transient arrest of cells at mainly the two cell cycle check-points, the G1/S and G2/M transitions. The first check-point in late G1 registers cell size, nutritional sate, and DNA damage. The second check-point is located in the order within G2 and M and it registers completion of DNA synthesis in G2 and preparatory steps for mitosis (Kuhl and Rensing, 2000). As mentioned earlier, DNA synthesis is rapidly inhibited by heat shock and results in DNA strand breaks. Disrupting the initiation of replication and mitosis represent the most important points of no return (Kuhl and Rensing, 2000). Sensitivity to heat shock is dependent on cell type and cell-cycle phase and depends on the severity of the stress. For example, Nover (1991) explained that there is an apparent difference to sensitivity to thermal killing between cells in the G1/G0 phases (more resistant) vs. those in G2/S phase (more sensitive). However, Coss et al. (1996) and Wachsberger and Coss (1990) showed that heat-induced cytoskeletal alterations play a role in the death of cells heated in G1 phase, but had no significant influence on the death of cells heated in the S phase in CHO cells.

Cellular Proteins

Heat shock rapidly increases the activation of a specific set of proteins, the heat shock proteins, which are tightly involved in the stress response. However, other protein

synthesis is normally inhibited by heat shock (Burdon, 1987; Kuhl and Rensing, 2000; Hilderbrandt et al., 2002) by inducing translational changes in cells. In Drosophila cells, the transcriptional regulation of heat shock protein genes is promoted while the translation of pre-existing mRNAs is repressed (Burdon , 1987). In mammalian cells, however, no sequestration of pre-existing mRNAs occurs but rather there is decrease in polysomes. Burdon (1987) explains that two possible reasons for the inhibition and loss of polysomes include a) inactivation of eIF-4F function, and b) dephosphorylation of ribosomal protein S6. In addition, heat shock induces degradation of damaged proteins by the ubiquitin-proteosome and lysosomal pathways (Kuhl and Rensing, 2000; Hilderbrandt et al., 2002) and losses of stable RNA occur as a result of increases in lysosomal ribonuclease activity (Burdon, 1987).

The changes in cellular physiology after exposure to elevated temperature have been referred to as the heat shock (or stress) response. The heat shock response is of fundamental importance to the cell's survival and is one of the most highly conserved genetic systems known. This response was discovered in 1962 by Ritossa, who found that heating salivary cells of Drosophila induced areas of localized transcription that correlated to increased synthesis of several families of proteins. These proteins were termed heat shock proteins (Hsps) due to their initial discovery in cells exposed to elevated temperatures. In addition to the increase in Hsps, the heat shock response also involves cytoskeleton reorganization, alteration in protein phosphorylation, and, at high temperatures, suppression of general protein synthesis (Coss and Linnemans, 1996). The importance of the heat shock response for cellular survival after heat shock has been demonstrated in many cells through experimental manipulation to cause induced

thermotolerance. In this phenomenon, cells are made resistant to the lethal effects of a severe heat shock by pre-exposure to a mild, sub-lethal elevation in temperature (De Maio, 1999). The phenomenon of induced thermotolerance involves increased production of Hsps.

Heat shock proteins, which are present in the cytoplasm, mitochondria, endoplasmic reticulum, and nucleus, have been divided into families that are named principally on the basis of the approximate molecular weight (kDa) of the protein. Heat shock protein families include Hsp100, Hsp00, Hsp70, Hsp60, Hsp40, and a large family of small heat shock proteins (sHsp) that range from 12-30 kDa (Knowlton, 1994). Inhibition of synthesis of Hsp70 and Hsp27 (Jakubowicz-Gil et al., 2002) by administration of antisense mRNA has been shown to prevent induced thermotolerance in specific cell types. In addition, transfection of rat cells with recombinant human Hsp70 conferred resistance to heat (Li et al., 1991) and constitutive high levels of Hsp27 give elevated intrinsic thermal resistance in rodent cells (Landry et al., 1989; Lavoie et al. 1993). Hsp27 (Arrigo, 1998) and Hsp32 are known to confer protection against other stressful stimuli (Bechtold and Brown, 2003). Antioxidant systems may also be involved in induced thermotolerance since inhibition of GSH synthesis prevented induced thermotolerance (Mitchell et al., 1983; Konings and Penninga, 1985; Skibba et al., 1989).

In general, Hsps function in the cell as molecular chaperones (Becker and Craig, 1994; Morimoto et al., 1992; Buchner, 1999), i.e., they function by mediating non-covalent associations and modifications of other proteins through associative interactions. It is now clear, in fact, that Hsps are not simply stress proteins but function as part of the normal machinery of the cell in the absence of cellular stress. Proteins in the Hsp90

family form heterodimers with other proteins to participate in formation of clathrin-coated pits during endocytosis, stabilization of steroid receptor and regulation of eIF2 kinase (Caplan, 1999). The Hsp70 family (Li and Mivechi, 1999), which are functional ATPases, can be divided into four types of proteins, namely Bip, found in the endoplasmic reticulum and also called glucose-regulated protein 70 since glucose concentration rather than heat shock is the primary regulator of transcription; mitochondrial Hsp70; Hsc70, a constitutive form that is expressed in high amounts in the absence of heat shock which can also be enhanced by heat shock; and Hsp70, the stress-inducible form that is present in low amounts in the absence of heat shock and whose synthesis is greatly increased during heat shock (Lee et al., 1992; Cheng and Lai, 1994; Macouillard-Poulletier de Gannes et al., 1998; Wang et al., 1998; Bechtold and Brown, 2003). Proteins of the Hsp70 family play a major role in protein folding. Among their actions, Hsc70 participates in ensuring that proteins are folded into an active conformation as they are being synthesized (Li and Mivechi, 1999). Both Bip and mitochondrial Hsp70 assist proteins to cross plasma membranes by facilitating unfolding and then refolding after membrane translocation. The Hsp60 (also known as chaperonins) are involved in Hsp70 and 90 interactions. The Hsp40 (Hdj-1) protein assists with ATP hydrolysis functions of Hsp70 (Michels et al., 1997) by stabilizing the ADP bound state of Hsp70 and thereby stimulating Hsp70 nucleotide hydrolysis, and by coupling nucleotide binding and hydrolysis to release of the substrate in a folded state (Minami et al., 1996).

Apart from their actions during normal cellular physiology, Hsps are involved in protecting the cell from harmful stimuli. The most studied Hsp family is Hsp70. Hsc70

is constitutively expressed with very little heat inducibility while Hsp70 is highly inducible by stress but is rarely present in unstressed cells (Widelitz et al., 1987; Wang et al., 1998; Macouillard-Poulletier de Gannes et al., 1998). During heat shock, Hsp70 is involved in recognizing denatured proteins and refolding them (Kiang and Tsokos, 1998). In addition, Hsp70 has been shown to have antiapoptotic properties (Mosser et al., 2000). Hsp32 is thought to contribute to cell recovery from stress through stimulating production of bilirubin, a strong antioxidant (Dalton et al., 1999; Bechtold and Brown, 2003).

In addition, many Hsps have a close relationship with the cytoskeletal components of the cell (Liang and MacRae, 1997). Several Hsp70 proteins have been characterized as microtubule-associated proteins (MAPs) and attach polymerized tubulin at the carboxy-terminal end. In addition, Hsps modulate actin assembly. Many studies have demonstrated that upon heat shock the inducible form of Hsp70 localizes to the cytoskeleton (Lee et al., 1992; Sanchez et al., 1994; Liao et al., 1995; Cheng and Lai 1994; Liang and MacRae, 1997; Wang et al., 1998). Thus, localization of Hsp70 to these sites could be a an important mechanism by which Hsp70 aids in cell recovery from heat shock. Furthermore, Hsp27 also have been shown to interact with the actin cytoskeleton during heat shock (Lavoie et al., 1993).

Elevation in temperature causes a rapid induction of Hsps (Lee et al., 1992; Cheng and Lai, 1994; Macouillard-Poulletier de Gannes et al., 1998; Wang et al., 1998; Bechtold and Brown, 2003). In the mammalian cell, Hsp70 is maintained in an inactive state by the transcription factors heat shock factor 1 and 2 (HSF1 and 2; Morimoto et al., 1992; Sistonen et al. 1994; Pirkkala et al. 2001). These transcription factors are synthesized constitutively in a latent form and undergo changes in respond to stressful

stimuli (HSF1) or to developmental and differentiation cues (HSF2) that allow the factors to stimulate transcription. For the case of heat shock, protein denaturation in the cell results in exposure of protein motifs in the hydrophobic regions of the denatured protein that is recognized by Hsp70. As a result, Hsp70 binds to the protein by a mechanism dependent upon ATP hydrolysis and interacts with the protein to refold it. At the same time, HSF1 is released from Hsp70 and forms homotrimers. The trimerized HSF1 translocates to the nucleus where it binds to heat shock elements (HSE) and, upon phosphorylation, directs transcription of the Hsp70 gene and other genes containing an HSE (Morimoto et al., 1992). Following transcription, the trimeric forms of HSF dissociate from the DNA and are converted back into non-active monomers (Morimoto et al., 1992).

The HSE is found in the promoter region of many genes induced by heat shock and consists of 5'-nGAAn-3' pentanucleotide repeats which usually occur in strings of four or more (Barros et al., 1992). Other regulatory elements also exist in the promoter regions of Hsps genes (Morimoto et al., 1992; De Maio, 1999). The Hsp70 gene also has a TATA box and a CAAT box upstream of the HSE (Morimoto et al., 1992). Furthermore, heat shock genes tend to lack introns or may possess them in small size and number. At least in Drosophila, the promoter region of the Hsp70 gene seems poised to induce rapid transcription upon heat shock. Transcriptional factors are bound and PolII is engaged and has transcribed 20-40 bases before it stops. After HSF binds to the HSE and gets phosphorylated PolII is activated and transcription of the gene occurs.

The chaperone complement in a cell determines the speed and duration of the heat shock response. Attenuation of the response is mediated by molecular chaperones Hsp70

and Hdj1 (Hsp40; Minami et al., 1996) through binding to HSF and prevention of trimerization. Accordingly, overexpression of Hsp70 can represses the activation of HSF-1. Thus, HSF-1 and Hsp70 form a negative-feedback loop whereby the amount of transcriptional activation of the Hsp70 gene by HSF-1 is dictated by the amounts of free Hsp70. When Hsp70 utilization increases during heat shock, HSF-1 trimerization is facilitated and increased transcription of Hsp70 results. Heat shock binding protein 1 (HSBP1) is another protein that negatively regulates stress-induced HSF1 DNA binding and transcriptional activity (Satyal et al., 1998). HSBP1 selectively binds the HSF1 trimer and converts it to a monomer. Furthermore, association of HSBP1 with HSF trimers and with Hsp70 coincides with attenuation of the heat shock response (Satyal et al., 1998).

Free Radicals and Antioxidants

Oxygen radicals and other reactive oxygen species (ROS) are formed as the result of incomplete reduction of molecular oxygen (Sies and Cadenas, 1985; Halliwell and Gutteridge, 1990). The major species of free radicals in cells are superoxide ion, hydrogen peroxide, and hydroxyl radical, which represent the result of reduction of O_2 with one, two or three electrons respectively. Reactive oxygen species are generated within a cell through several biochemical pathways including oxidative phosphorylation and actions of enzymes such as NADPH oxidase, xanthine oxidase, and cycloheximide (Guérin et al., 2001). It has been estimated that about 1-2% of the oxygen consumption of a cell is incompletely oxidized to form ROS (Fulbert and Cals, 1992).

Many conditions that disrupt cellular homeostasis enhance the production of reactive oxygen species (ROS; Flanagan et al., 1998; Ozawa et al., 2002). Because of

their strong electrophilic properties and propensity to react with most molecules within the cell, ROS are potent mediators of cellular injury (Kourie, 1998; Jabs, 1999; Leonarduzzi et al., 2000; Shackelford et al., 2000; Dizdaroglu et al., 2002; Halliwell, 2003). Cellular pathologies caused by ROS include damage to nucleic acids, which can disrupt transcription, translation and DNA replication (Dizdaroglu et al., 2002), oxidation and crosslinking of proteins, and lipid peroxidation (Wells et al., 1997). Lipid peroxidation is a particularly-harmful response to free radicals since in the presence of iron, the products of lipid peroxidation can generate additional hydroxyl radicals in a chain reaction that consumes unsaturated fatty acids in membranes (Dargel, 1992). Changes in lipids and membrane proteins induced by ROS can result in changes in intracellular calcium concentrations (Dalton et al, 1999) and ion transport mechanisms (Kourie, 1998).

Flanagan et al. (1998) demonstrated that heat shock increases the flux of cellular free radicals in a non-transformed rat intestinal epithelial cell line. Their work supports the hypothesis that heat shock-induced cellular damage may be mediated by the oxidative stress resulting from increased generation of ROS. Elevated temperatures also activate enzymes which are involved in the generation of free radicals. For example, heat shock increases the conversion of xanthine dehydrogenase to the oxidase form, an important source of oxygen-derived free radicals (Skibba et al., 1989). Studies by Skibba et al. (1989) and Powers et al. (1992) showed that the hyperthermic toxicity to the liver is the result of oxidative stress brought about by conversion of xanthine oxidase in hyperthermic liver perfusions. In addition, liver levels of GSH were significantly lowered following perfusion at hyperthermic temperatures. Further, heat shock has been

reported to increase phospatidilinositol turnover and to release arachidonic acid, the substrate for cyclooxygenase and lipooxygenase which are possible sources of ROS (Stevenson et al., 1986).

Given the abundant production of ROS in cells (~1-2% of oxygen consumed; Fulbert and Cals, 1992), it is not surprising that cells possess several biochemical systems to remove free radicals. In general, antioxidant systems can be divided into those that react directly with free radicals to reduce the molecule to water and to enzymes that facilitate transfer of electrons from donor molecules to achieve reduction of the free radical. Among the former are water soluble antioxidants such as GSH and ascorbic acid that are functional in the cytoplasm, and lipid-soluble antioxidants such as vitamin E (Kaikkonen et al., 2001) and β-carotene (Zhang and Oaye, 2000) that function in lipid membranes. Among the enzymes involved in antioxidant defense are superoxide dismutase (Harris, 1992), which converts superoxide to hydrogen peroxide, catalase and GSH peroxidase (Arthur, 2000), which reduce hydrogen peroxide to water. Glutathione is a tripeptide with sequence γ-glu-cys-gly in which the hydrogen on the sulfhydryl of the cysteine is used as an electron donor (Meister, 1983). Reduced GSH can be regenerated from oxidized GSH by the enzyme GSH reductase and NADPH.

Several studies have demonstrated a beneficial effect of antioxidants on survival or recovery of cells after heat shock. Addition of the antioxidant taurine blocked the heat shock-induced killing of lymphocytes (Malayer et al., 1992). In another study, Kuninaka et al. (2000) showed that suppression of mitochondrial manganese superoxide dismutase in HCT116 colon cancer cells by antisense RNA increased sensitivity to hyperthermia in a dose-dependent manner. When HeLa cells are pretreated with diethyl

dithiocarbamate to deplete cells of super oxide dismutase or with diethymaleate, to deplete cells of non-protein thiols, the inhibitory effects of heat were more pronounced (Evans et al., 1983; Freman et al., 1985).

Heat Shock-Induced Apoptosis

Apoptosis is a form of programmed cell death essential for normal development and homeostasis as well as in the removal of damaged cells. Among the features of apoptotic death are the generation of large condensed chromatin bodies and degradation of DNA into nucleosomal units of 200 base pairs fragments, condensation of cytoplasm, generation of evoluted membrane segments, cell fragmentation and shrinkage, and disintegration of mitochondria (Fiers et al., 1999; Gulbins et al., 2000). Apoptosis initiation may be brought about by many physiological and pathophysiological stimuli including heat shock (Luchetti et al., 2002; Ko et al., 2000; Paula-Lopes and Hansen, 2002a,b).

Apoptosis involves the activation of caspases that cleave a variety of subcellular targets to cause the cellular features of apoptosis described above (Gulbins et al., 2000). The degradation of DNA into 200 bp nucleosomal units that is the hallmark of apoptosis involves activation of caspase 3 from an inactive proenzyme form and the result proteolysis of an inhibitor to caspase-induced DNAse as well as the DNA repair enzyme PARP (poly(ADP-ribose) polymerase; Earnshaw et al., 1999). One of the two major mechanisms whereby caspases become activated is through increased permeability of the mitochondria to release pro-apoptotic proteins such as cytochrome c, apoptosis-inducing factor (AIF), and smac/Diablo from the inter-membrane space of the mitochondria (Ichas and Mazat, 1998; Duchen, 1999; Waterhouse et al., 2003). When mitochondria are

damaged by harmful stimuli, the proton gradient is lost and the mitochondrial matrix undergoes swelling (Ichas and Mazat, 1998). This swelling phenomenon is known as permeability transition and results in outer mitochondrial membrane rupture and release of proteins normally housed in the inter-membrane space (Duchen 1999; Waterhouse et al., 2003). Cytochrome c induces caspase activation by forming a complex called the apoptosome with a protein called Apaf-1 (apoptosis activating factor 1; Jiang and Wang, 2000). Upon formation of the apoptosome Apaf1 undergoes a conformational change which leads to the autoproteolysis and self activation of activate procaspase-9. Mature caspase-9 cleaves procaspase-3 to form caspase-3, the main executioner of apoptosis (Gottlieb, 2000).

Environmental insult, such as heat shock, will lead to the initiation of the apoptotic cascade through actions directly on the membrane. Acid sphingomyelinase will be activated by heat shock and in turn generate ceramide from sphingomyelin, which through actions on the stress activated protein kinase/c-Jun N-terminal kinase (SNAP/JNK) activates caspases Heat shock induces apoptosis in many cells (Boreham et al., 1997; Matsumo et al., 1997; Buzzard et al., 1998; Jakubowicz-Gil et al., 2002). For example, Jakubowicz-Gil et al. (2002) showed that heat shock at 42.0°C for 1 h increased induction of apoptosis in HeLa cells as estimated by comet assay. In addition, heat shock induces changes in the mitochondrial membrane potential and cytochrome c release resulting in apoptosis (Ko et al., 2000). Heat-induced chromatin changes characteristic of apoptosis have been characterized in HL60 human hemopoietic cells (Luchetti et al., 2002). Heat shock of 43.0°C for 1 h induced the characteristic oligonucleosomic fragmentation as determined by DNA gel electrophoresis. In addition, chromatin clusters

at the nuclear periphery, frequently showing dense masses protruding towards the cytoplasm were observed.

Responses of Preimplantation Embryos to Heat Shock

Disruption of Cellular Function

While many experiments have demonstrated that exposure to elevated temperature can inhibit development of preimplantation embryos (Ealy and Hansen, 1994; Ealy et al., 1995; Edwards et al., 1997; Aréchiga and Hansen, 1998; Ju et al., 1999; Al-Katanani and Hansen, 2002; Block et al., 2002; Krininger et al., 2002; Paula-Lopes et al., 2003a,b) there is little experimental evidence to describe the mechanisms by which this inhibition is affected. An early study by Elliot and Ulberg (1971) indicated that heat shock can compromise formation and function of nucleoli. Four- and eight-cell mouse embryos were collected from control or heat-stressed females and cultured in the presence of [^{3}H]uridine for 1 h. Embryos collected from heat-stressed females had fewer stained nucleoli than did those from controls, indicating a reduced RNA synthetic ability. Furthermore, there were fewer nucleoli in heat-stressed embryos than in control embryos.

There are also data that heat shock can reduce protein synthesis in embryos. In particular, exposure of mouse (Edwards et al., 1995) and bovine embryos to a heat shock of 42.0°C decreased total intracellular protein synthesis as determined by monitoring incorporation of [^{35}S]methione into trichloacetic acid-precipitable protein. In bovine embryos, the effects of heat shock depended on stage of development (Edwards and Hansen, 1997). While heat shock reduced protein synthesis from the two-cell stage through blastocyst stage of development, it had no effect on protein synthesis by

expanded blastocysts and increased protein synthesis by hatched blastocysts (Edwards and Hansen, 1997).

At least in the mouse, actions of heat shock on embryos involve free radicals. Maternal heat stress significantly increased hydrogen peroxide concentration of two-cell embryos (Ozawa et al., 2002) while intracellular concentrations of GSH were reduced by heat shock in two-cell embryos (Ozawa et al., 2002) and morulae (Aréchiga et al., 1995).

Adverse effects of heat shock on mouse embryonic development can be minimized by various antioxidants (Malayer et al., 1992; Aréchiga *et al.,* 1994). For example, some thermoprotection was provided to mouse embryos by addition to culture medium of taurine (Malayer et al., 1992), GSH (Aréchiga et al., 1994, 1995) and vitamin E (Aréchiga et al., 1994). In contrast, bovine embryos in general are not protected from effects of heat shock by antioxidants. While taurine and GSH provided some thermoprotection to blastocysts (Ealy et al., 1992), there was no protective effect of GSH, GSH ester, taurine or vitamin E (Ealy et al., 1995; Paula-Lopes et al., 2003a) on embryos at earlier stages of development. Taken together, these results indicate that free radicals are a more important source of cellular damage in mouse embryos than is the case for bovine embryos.

Thermoprotective Mechanisms in Embryos

Hsp70

Heat shock proteins are one of the first proteins to be synthesized constitutively in the early preimplantation embryo (Christians et al., 1995). In addition, heat shock can induce synthesis of Hsp70 in mouse (Edwards et al., 1995) and bovine embryos (Edwards and Hansen, 1996; Edwards et al., 1997; Chandolia et al., 1999a; Kawarsky and King,

2001). Data are contradictory, however, as to how early in development heat shock can first induce Hsp70 synthesis. Edwards and Hansen (1996), Edwards et al. (1997), and Chandolia et al. (1999a) all reported a large increase in the synthesis of Hsp70 following heat shock in the two-cell bovine embryo, a stage at which the embryo's genome is largely inactive (Thompson, 2000). The increase in Hsp70 synthesis was due to transcription since inhibitors of transcription reduced the amount of Hsp70 mRNA (Chandolia et al., 1999a). In contrast, Kawarsky and King (2001) did not observe induction of Hsp70 synthesis until the eight-cell stage. In porcine embryos, there was no effect of heat shock on synthesis of Hsp70 or Hsp90 in compact morula or blastocysts (Kojima et al., 1996).

Antioxidants

As described earlier, heat shock appears to increase free radical production in embryos, at least in mice (Aréchiga and Hansen, 1995; Ozawa et al., 2002). Although inhibition of GSH synthesis by buthionine sulfoximine (BSO) did not alter the susceptibility of morula-stage mouse embryos to heat shock (Aréchiga et al., 1998), it did block the induced thermotolerance phenomenon (Aréchiga et al., 1995). Moreover, induction of GSH synthesis by S-adenosyl-L-methionine decreased the deleterious effects of heat shock (Aréchiga et al., 1995).

Apoptosis

Recent studies demonstrate that early bovine embryos are capable of undergoing heat-induced apoptosis (Krininger and Hansen, 2002; Paula-Lopes and Hansen, 2002a,b). The response of embryos to heat shock is developmentally regulated since embryos less than the 8-16 cell stage were not capable of undergoing apoptosis in response to heat

shock (Paula-Lopes and Hansen, 2002a). There are also developmental changes in induction of apoptosis by exposure to arsenic (Krininger and Hansen, 2002) and tumor necrosis factor-α (Soto et al., 2003), with 2-cells being incapable of apoptosis and embryos at Day 5 after insemination undergoing apoptosis in response to these stimuli.

When induced, not all blastomeres of the bovine embryo undergo apoptosis. After 41.0°C for 9 h, for example, 15% of cells from a Day 5 embryo were apoptotic. While apoptosis is obviously fatal to the cell undergoing it, it is possible that a limited amount of apoptosis in response to a harmful stimulus is beneficial to the function of a tissue or, in this case, embryo. This argument has been advanced for the preimplantation embryo exposed to heat shock and experimental evidence obtained to support it. First, embryos that are not capable of apoptosis (< 8-16 cells) are more susceptible to inhibition of development by heat shock than embryos capable of apoptosis (Edwards et al., 1997). Furthermore, inhibition of heat shock-induced apoptosis by incubation with the caspase inhibitor z-DEVD-fmk not only blocked apoptosis in Day 5 bovine embryos but also exacerbated the block to development cause by heat shock (Paula-Lopes and Hansen, 2002b).

Synopsis - Rationale for Thesis

Although there is a great deal of information on the effects of heat shock on cellular systems, information is scarce on the effects of heat shock on early mammalian embryo. Most of the work performed to understand the biochemical, cellular and ultrastructural effects of heat shock on cells have involved temperatures that would be lethal in most mammals. For example, temperatures in the range of $> 43.0°C$ have been used to study the cytoskeleton (Wachsberger and Coss, 1990), functional morphology of organelles (Funk et al., 1999), and mitochondrial function (Macouillard-Poulletier de Gannes et al., 2000). In contrast, embryonic loss in females due to heat stress occurs when maternal rectal temperatures are in the range of 40.0-41.0°C in cattle (Dunlap and Vincent, 1971; Putney et al., 1988; Ealy et al., 1993), 39.0-40.3°C in mice (Elliot et al., 1968; Elliot and Ulberg, 1971;Bellvé, 1972), 40.0°C in rats (Fernandez-Cano, 1958) and 40.0°C in sheep (Alliston and Ulberg, 1961; Alliston et al., 1961; Dutt, 1963). Hence, many of the conclusions regarding the mechanism by which heat shock damages cells derived from studies using temperatures at 43.0°C or above may not be relevant to the situation for the early mammalian embryo. Accordingly, the objective of this dissertation is to characterize some of the cellular, subcellular and developmental responses of the two-cell bovine embryo (i.e., when the embryo is most sensitive to heat shock in the cow) to a physiologically-relevant heat shock. These results offer the promise of extending our understanding of cellular consequences of heat shock to the conditions that are actually experienced commonly by mammalian cells. In addition, identification of the

mechanisms by which heat shock disrupts embryonic development may eventually lead to new approaches for improving embryonic survival during heat stress through manipulation of cellular function of the embryo.

CHAPTER 2
DEVELOPMENT OF CULTURED BOVINE EMBRYOS AFTER EXPOSURE TO INCREASED TEMPERATURES IN THE PHYSIOLOGIC RANGE

Introduction

Heat stress can reduce fertility in cattle (Dutt, 1963; Dunlap and Vincent, 1971; Turner, 1982; Ealy et al., 1993; Ryan et al., 1993) and other species (Shah, 1956; Alliston and Ulberg, 1961; Tompkins et al., 1967). The mechanism by which heat stress causes embryonic mortality is multifactorial since heat stress can alter several aspects of reproductive physiology including blood flow to the reproductive tract (Roman-Ponce et al., 1978), ovarian steroid concentrations (Gwazdauskas et al., 1973; Roman-Ponce et al., 1981; Badinga et al., 1993; Trout et al., 1998), and patterns of follicular development (Badinga et al., 1993; Wolfenson et al., 1995). One possibility is that embryos cannot survive the elevation in oviductal and uterine temperature coincident with heat stress. Culture of embryos at elevated temperatures has been reported to reduce embryonic development (Ulberg and Sheean, 1973; Edwards and Hansen, 1996; Edwards and Hansen, 1997). Similarly, elevated culture temperatures can compromise oocyte function (Lenz et al., 1983; Baumgartner and Chrisman, 1987; Edwards and Hansen, 1996) and fertilization rate (Lenz et al., 1983; Ulberg and Burfening, 1967).

Studies in cattle that have demonstrated deleterious effects of heat shock on cultured embryos have used temperatures of 41.0° through 43.0°C (Ealy et al., 1992; Ealy and Hansen, 1994; Edwards and Hansen, 1996; Edwards and Hansen, 1997). However,

these experimental temperatures are higher than those often experienced by heat-stressed cows with reduced fertility. For example, Dunlap and Vincent (1971) saw a reduction in fertility from 48% in control animals to 0% for heifers exposed to 32.2°C for 72 h immediately following breeding. In that study, however, the average rectal temperature of heat-stressed heifers was 40.0°C. A heat shock of 40.0°C actually increased development of cultured bovine embryos (Ryan et al., 1992).

Heat shock in culture also differs from the situation in utero in several other respects. In vitro, for example, increases in temperature cause decreased CO_2 solubility in culture medium and an increase in medium pH. This confounding of pH with heat shock has not been considered heretofore in experiments evaluating effects of heat shock on embryos or, more generally, for cultured cells. Experiments on heat shock have also been done in a gaseous environment where O_2 content is higher than in the oviduct (i.e. 8.7% in rabbit; Fischer and Bavister, 1993). Since effects of heat shock on embryonic development also involve free radicals (Ealy et al., 1992; Aréchiga et al., 1995), it is unknown whether heat shock would affect embryonic development at oxygen environments similar to those in vivo.

The objectives of the present studies were: 1) to determine whether exposing bovine oocytes and embryos to temperatures characteristic of body temperatures of heat-stressed cows would affect embryonic development in vitro, 2) to test if culturing early bovine embryos in low oxygen concentration would eliminate the adverse effects of heat shock on embryonic development, and 3) to verify that the detrimental effects of heat shock were not due by changes in pH as a result of reduced solubility of CO_2 at increased temperatures.

Materials and Methods

Materials

Follicle stimulating hormone (FSH), was Folltropin®-V from Vetrepharm Canada Inc. (London, Ontario) and was purchased from AgTech (Manhattan, KS). The culture media SP-TL, IVF-TL, and HEPES-TL were prepared by Cell and Molecular Technologies, Inc. (Lavallete, NJ) using recipes described by Parrish et al. (1986). Using these media, SP-TALP (Tyrodes Albumin Lactate Pyruvate), HEPES-TALP, and IVF-TALP were prepared as described by Parrish et al. (1986). The CR1aa medium was prepared as described by Rosenkrans et al. (1993). Bovine serum albumin Fraction V (Fraction V BSA), essentially fatty-acid free BSA Fraction V (EFAF-BSA), and all other chemicals were from Sigma (St. Louis, MO).

In Vitro Production of Embryos

Bovine ovaries were obtained from a local abattoir located at a travel distance of ~1.5 h from the laboratory. Ovaries were transported to the laboratory in 0.9% (w/v) NaCl at room temperature. Ovaries were sliced and oocyte-cumulus complexes (OCCs) were collected into a beaker containing oocyte collection medium [TCM-199 with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U/ml heparin), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin]. Oocyte-cumulus complexes were cultured in 50 µl microdrops of oocyte maturation medium (TCM-199 with Earle's salts supplemented with 100 IU/ml penicillin, 0.01 mg/ml streptomycin, 2 µg/ml estradiol, 20 µg/ml FSH, and 0.2 mM sodium pyruvate) in groups of 10 for 20-23 h. After maturation, OCCs were washed in HEPES-TALP and placed in groups of ~ 30 in 600 µl IVF-TALP in 4-well plates. Spermatozoa, purified by Percoll

gradient centrifugation (Parrish et al., 1986) and suspended in SP-TALP, were added to the matured oocytes at a density of ~1 x 10^6 spermatozoa/well. Coculture of spermatozoa and OCCs proceeded for 8-10 h after which putative zygotes were denuded of cumulus cells by vortexing in a 2.0 ml microcentrifuge tube containing ~0.5 ml HEPES-TALP for 5 min. Putative zygotes were cultured in 50 µl microdrops of CR1aa. On Day 5 post-insemination, 5 µl fetal bovine serum were added to each culture drop.

All cultures were performed at 38.5°C in 5% (v/v) CO_2 in humidified air unless otherwise specified. When cultures were performed at elevated temperatures, the CO_2 percentage of the gas phase was adjusted to prevent pH changes in medium caused by decreased solubility of CO_2 at increased temperatures. The CO_2 percentage needed to maintain a pH of ~7.4 in the CR1aa medium was experimentally determined for each temperature. These were 5.5%, 6.0%, 6.5%, and 7.0% CO_2 for 39.5, 40.0, 40.5, and 41.0°C, respectively. The accuracy of the incubators was ± 0.2°C for one of the incubators and 0.1°C for two of the incubators. The temperatures of all incubators were routinely calibrated by the use of mercury thermometers.

Heat Shock During Fertilization

Co-culture of spermatozoa and OCCs progressed for 8 h at 38.5, 40.0, or 41.0°C. After fertilization, putative zygotes were cultured at 38.5°C for 8 d. Cleavage rate was determined on Day 3 post-insemination and development to the blastocyst stage was determined on Day 8 post-insemination. This experiment was replicated six times using a total of 179-180 oocytes per treatment.

Heat Shock at the One-Cell Stage

After co-culture of spermatozoa and OCCs had taken place (8 hpi), putative zygotes were cultured in 50 µl microdrops in groups of 27-32 at 38.5°C continuously or were exposed to 40.0 or 41.0°C for 3, 6, 9, or 12 h. After this period, all embryos were cultured at 38.5°C for the duration of the culture. Cleavage rate was determined on Day 3 post-insemination and development to the blastocyst stage was determined on Day 8 post-insemination. The experiment was replicated four times using 117-121 putative zygotes per treatment.

Heat Shock at the Two-Cell Stage

Two experiments were conducted. In both, embryos at the two-cell stage were collected from culture drops at ~ 28 h post-insemination and transferred to a new drop of CR1aa medium (8-25 embryos/drop). For the first experiment, embryos were then cultured for 3, 6, 9, or 12 h at 40.0 or 41.0°C or were maintained at 38.5°C. Further, a group of embryos was left undisturbed in the original culture drops (i.e., two-cell embryos were not separated) to test whether the handling itself would adversely affect development. After heat shock, embryos were returned to 38.5°C for the duration of culture. Development to the blastocyst stage was determined on Day 8 post-insemination. Unlike most experiments, the type of BSA used to supplement CR1aa was Fraction V. The experiment was replicated five times with a total of 54-84 two-cell embryos per treatment.

The second experiment was conducted similarly except that heat shocks were 39.5 and 40.5°C. Further, the CR1aa was supplemented with EFAF-BSA. The experiment was replicated four times with a total of 52-68 embryos per treatment.

Effect of Type of BSA on Response to Heat Shock

The experiment was designed as a 2 x 2 factorial with two types of BSA (Fraction V and EFAF) and two temperatures (38.5 or 41.0°C). Two-cell embryos were collected at ~28 h post-insemination and placed in CR1aa supplemented with either EFAF-BSA or with Fraction V BSA. Embryos were cultured at 38.5°C continuously or at 41.0°C for 12 h and 38.5°C thereafter. Development to the blastocyst stage was determined on Day 8 post-insemination. The experiment was replicated three times with a total of 57-58 embryos per treatment.

Effect of Oxygen Concentration on Response to Heat Shock

This experiment was performed to test whether the effect of heat shock in culture is exacerbated by a high O_2 environment. In order to regulate the gaseous environment of cultured embryos, a chamber was constructed (Figure 2-1) from a 115 ml vacuum filter unit from Corning. Tygon tubing (3.2 mm i.d. x 6.4 mm o.d.) with a stopcock fastened to the end was attached to the hose connector and a 00 size rubber stopper was inserted into the pour spout. The cellulose nitrate membrane in the filter was punctured in several places to facilitate movement of gases. Culture plates were gently positioned on top of the membrane. The lid of the filter system was taped securely with scotch tape and parafilm was placed around the lid to make the chamber airtight. Gases where injected for 3 min through the pour spout from a hose connected to the gas cylinder while the stopcock was in the open position. After injection of gases was completed, the rubber stopper was fastened to the pour spout and the stopcock was dialed to the closed position. To prevent contamination during injection of gases, a filter (0.22 μm) was attached to the

gas hose. The chambers were shown to be airtight since pH was maintained for 24 h in the presence of various gas mixtures.

The design was a 2 x 2 factorial with two O_2 tensions (5% or 20.95%) and two temperatures (38.5 and 41.0°C). Embryos were collected at the two-cell stage and placed in fresh microdrops (14-27/drop). Culture plates were then placed into airtight chambers. The chambers containing the embryos cultured at 38.5°C were injected witb a mixture of gases containing high (20.95%, v/v) or low (5%, v/v) O_2 with 5% (v/v) CO_2 and a balance of N_2. Chambers containing the embryos cultured at 41.0°C were injected with a similar mixture of gases except that CO_2 was 7% (v/v) to maintain a pH of ~ 7.4 and the N_2 content was adjusted accordingly. After 12 h of culture, plates were removed from the chambers and returned to incubators at 38.5°C and 5% (v/v) CO_2 in air until Day 8 post-insemination. The experiment was replicated four times with a total of 120-122 embryos per treatment.

Rectal Temperatures of Heat-Stressed Lactating Dairy Cows

This experiment was performed as a prelude to determining effects of culture temperatures characteristic of those experienced by heat-stressed cows on embryonic development. Animals used were primiparous (n = 8) or multiparous (n = 17) lactating Holsteins (50-150 d in milk except for one cow at 266 d in milk) located in north Florida (Hague, Florida; 29' 46" N 82' 25" W). Cows were milked three times per Day and received injections of bovine somatotropin (Posilac, Monsanto, Chesterfield, MO) according to manufacturer's recommendations. Milk yields on the day of recording ranged from 17.2 to 33.6 kg/d. Cows were maintained in free-stall barns equipped with a cooling system utilizing high-pressure foggers. On each of three separate days in August

Figure 2-1. Chamber used to regulate gaseous environment of cultured embryos in low and high oxygen tensions. The chamber was constructed from a 115 ml-filter system from Corning. Tygon tubing (3.2 mm i.d. x 6.4 mm o.d.) with a stopcock fastened to the end was attached to the hose connector and a 00 size rubber stopper was inserted into the pour spout. The cellulose nitrate membrane in the filter was punctured in several places to facilitate movement of gases. Culture plates were gently positioned on top of the membrane. The lid of the filter system was taped securely with scotch tape and parafilm was placed around on the lid to make the chamber airtight. Gases where injected for 3 min through the pour spout from a hose connected to the gas cylinder while the stopcock was in the open position. After injection of gases was completed, the rubber stopper was fastened to the pour spout and the stopcock was dialed to the closed position. To prevent contamination during injection of gases, a filter (0.22 μm) was attached to the gas hose. The chambers were shown to be airtight since pH was maintained for 24 h in the presence of various gas mixtures.

1999, a group of 9 cows was examined. Cows were chosen so that three cows were greater than 75% white in color (white), three cows were between 25-75% white (black and white) and three cows were less than 25% white (black). At each hour for 24 h, rectal temperatures were measured using mercury rectal thermometers (precision = 0.1°C) and respiration rates were determined. In addition, relative humidity, dry bulb temperature, and black globe temperature were recorded each hour. For each replicate, a separate group of 8 or 9 cows in a different free-stall barn was used.

Exposure of Embryos to a Pattern of Temperatures Similar to those Experienced by Heat-Stressed Cows

Incubators were set at 38.5, 39.5, or 40.5°C. The heat shock treatment consisted of sequential exposure to 38.5°C (5 h), 39.5°C (5 h), 40.5°C (5 h), and 39.5°C (9 h). Given the limited number of incubators available, this pattern was found to most closely mimic the pattern of rectal temperatures experienced by cows in the previous experiment (see results). Putative zygotes were cultured at either 38.5°C continuously (control), heat shocks from d 0-1 post-insemination (HSD0-1), or heat shock on each day from Day 0-8 post-insemination (HSD0-8). The experiment was replicated four times with a total of 121 to 122 embryos per treatment.

Statistical Analysis

For embryo experiments, percent cleavage and development was calculated for each microdrop of putative zygotes or embryos. Each experiment was replicated on several days with one or more microdrop per treatment on each day. Data were analyzed by least-squares analysis of variance using the PROC GLM procedures of SAS (1989). All main effects were considered fixed. Embryo number per drop was also included as a covariate for experiments with two-cell embryos where there was variation in the number

of embryos per drop. Percentage data were transformed by arcsine transformation before analysis. The analysis of transformed data was used for probability values but least-squares means ± SEM are presented from analysis of untransformed data. In some experiments, the SAS option pdiff was used to compare the mean of each treatment with the mean of the control group. Also, orthogonal contrasts were used to determine whether the effects of duration of heat shock followed a linear, quadratic or cubic pattern.

Data from rectal temperatures were analyzed by least-squares analysis of variance. The mathematical model included main effects of coat color, replicate, cow (replicate x coat color), and time. All interactions were included in the model. Cow was considered a random effect and other main effects were considered fixed.

Results

Effect of Heat Shock During Fertilization

Bovine oocytes fertilized at 41.0°C had lower ($P < 0.01$) cleavage rate than oocytes fertilized at 38.5°C (Figure 2-2, top panel). The proportion of oocytes ($P < 0.01$) and cleaved embryos ($P < 0.01$) that developed to blastocyst was also decreased ($P < 0.01$) by 41.0°C (Figure 2-2, bottom panel). A heat shock of 40.0°C during fertilization had no effect on cleavage rate and tended to increase ($P < 0.06$) the proportion of oocytes forming blastocysts when compared to oocytes fertilized at 38.5°C.

Heat Shock at the One-Cell Stage

No reduction in cleavage rate or blastocyst formation was detected for one-cell embryos cultured at 40.0°C for 3, 6, 9, or 12 h or 41.0°C for 3 or 6 h. However, a heat shock of 41.0°C for 12 h reduced ($P < 0.01$) cleavage rate (Figure 2-3, top panel).

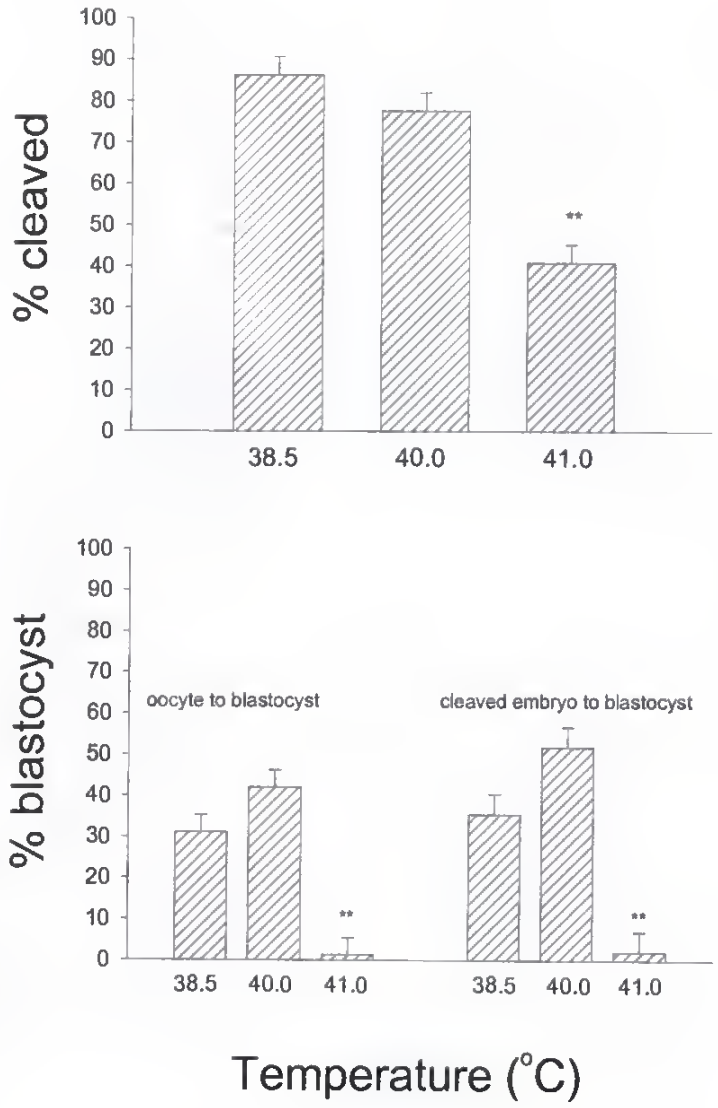


Figure 2-2. Effect of temperature during fertilization on the percentage of oocytes that cleaved (top panel) and that became blastocyst on Day 8 post-insemination (bottom panel). Data are least-squares means ± SEM. Fertilization was for 8 h at the temperatures indicated. Replications were 6 and number of oocytes per treatment were 179-180. ** = different from control, $P < 0.01$.

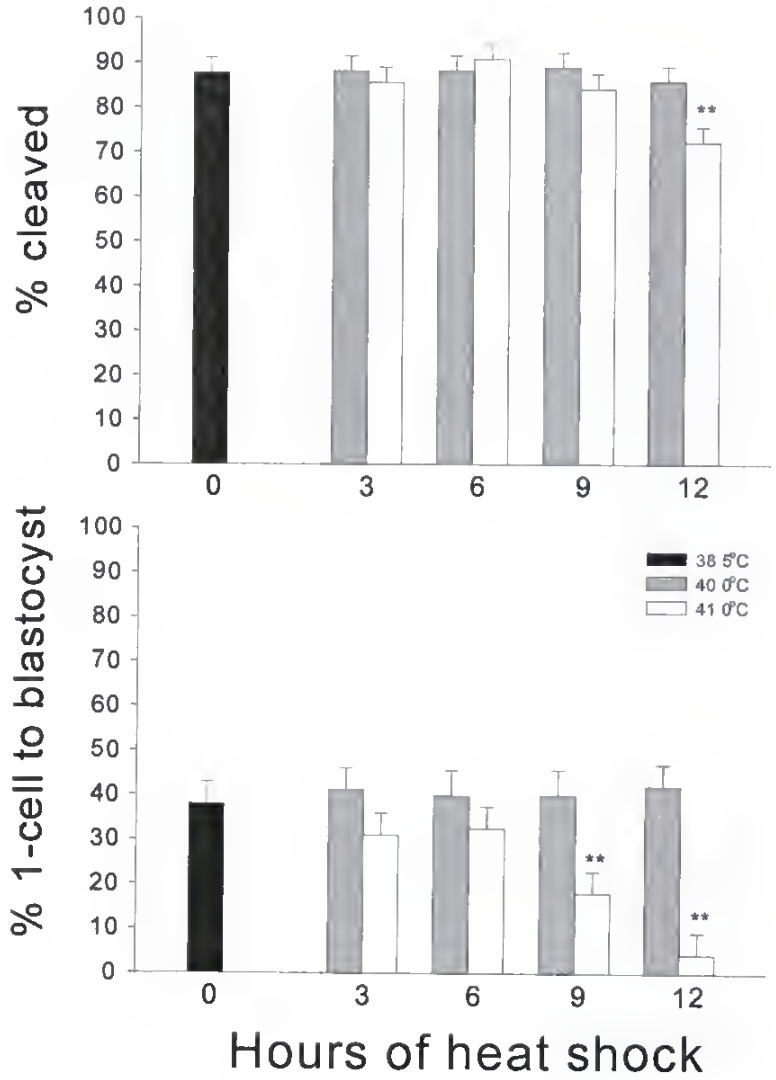


Figure 2-3. Effect of heat shock at the one-cell stage on the percentage of oocytes that cleaved (top panel) and that became blastocyst on Day 8 post-insemination (bottom panel). Data are least-squares means ± SEM. Replications were 4 and number of oocytes per treatment were 117-121. ** = different from control, $P < 0.01$.

Similarly, development to the blastocyst stage was adversely affected only by heat shocks of 41.0°C for 9 or 12 h ($P < 0.01$; Figure 2-3, bottom panel).

Heat Shock at the Two-Cell Stage

In the first part of this experiment, 40.0°C did not significantly reduce the percent of two-cell embryos that became blastocyst by Day 8 post-insemination (Figure 2-4, top panel). In contrast, 41.0° C for 9 and 12 h reduced ($P < 0.01$) the proportion of embryos that were blastocysts at Day 8 post-insemination. In the second experiment, neither 39.5 nor 40.5°C reduced the rate of blastocyst formation (Figure 2-4, bottom panel). Note that the proportion of embryos reaching blastocyst was greater for the second experiment, in which medium was supplemented with EFAF-BSA, than for the first experiment, in which medium was supplemented using Fraction V BSA. Accordingly, another experiment was done to verify this effect of BSA type, and to test whether BSA source would affect responses of heat shock (Figure 2-5, top panel). Two-cell embryos cultured at 38.5°C had a higher rate of blastocyst formation than embryos exposed to 41.0°C for 12 h ($P < 0.02$). In addition, the percent of embryos becoming blastocyst was higher ($P < 0.01$) for embryos cultured in CR1aa medium containing EFAF-BSA than for those cultured in CR1aa medium containing Fraction V. There was no temperature x BSA interaction; however, indicating that the effect of heat shock was similar for the two media.

Effect of Oxygen Concentration on Response to Heat Shock

Two-cell embryos cultured at 38.5°C had a higher rate ($P < 0.01$) of blastocyst formation than embryos exposed to a heat shock of 41.0°C for 12 h (Figure 2-5, bottom panel). Low O_2 had a beneficial effect on subsequent development to the blastocyst stage

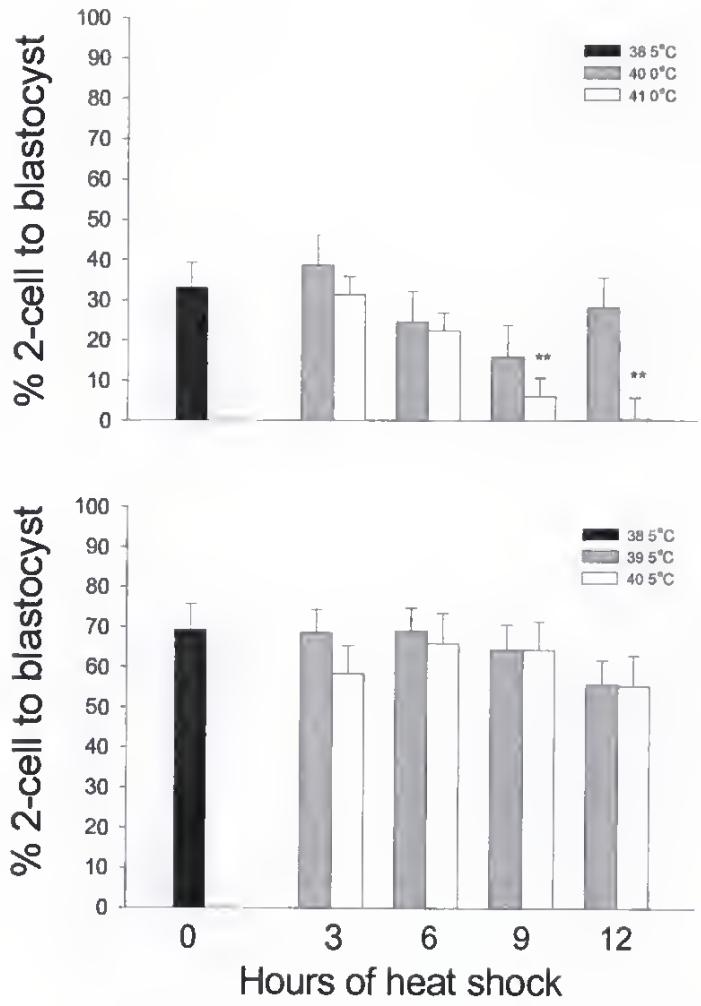


Figure 2-4. Effect of heat shock at the two-cell stage on the percentage of two-cell embryos that became blastocyst on Day 8 post-insemination. Embryos were shocked at various periods beginning at ~28 h post-insemination. Data are least-squares means ± SEM. For 40°C, there was a cubic effect ($P < 0.1$) of duration of heat shock. For 41.0°C, there was a linear effect ($P < 0.001$) of duration of heat shock. Replications were 4 and number of oocytes per treatment were 52-68. ** = different from control, $P < 0.01$.

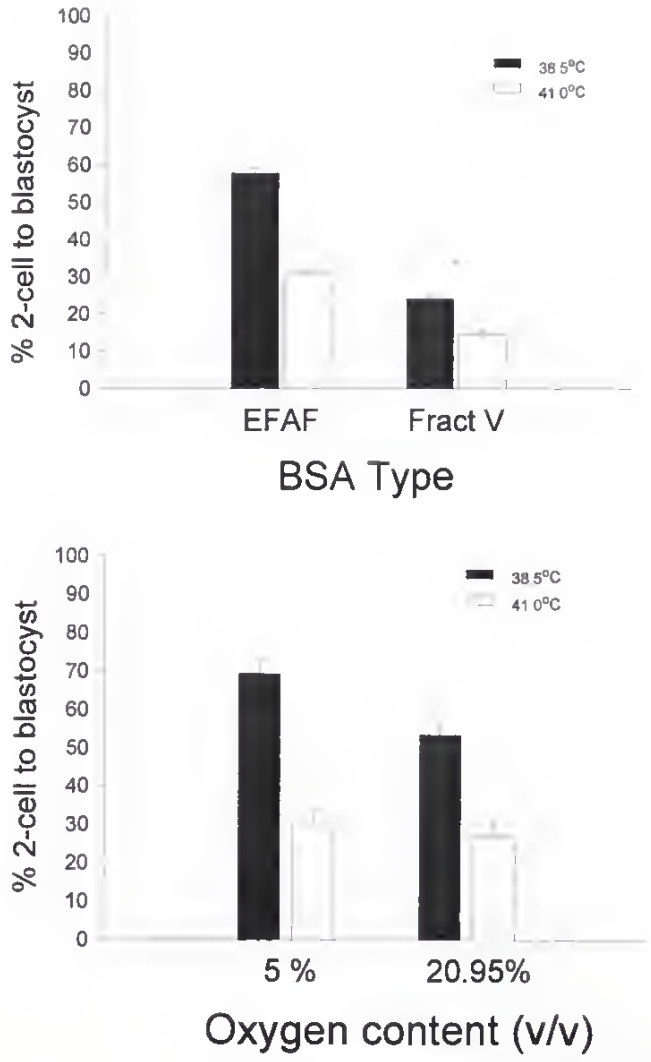


Figure 2-5. Top panel. Effect of culture conditions on responses of two-cell embryos to heat shock. Effect of type of bovine serum albumin (BSA) used to supplement the culture medium on subsequent development to the blastocyst stage. Results are the percentage of two-cell embryos that became blastocyst on Day 8 post-insemination. The percent of embryos becoming blastocyst was different ($P < 0.02$) between embryos cultured at 38.5°C vs. 41.0°C for 12 h and between embryos cultured in medium containing essentially fatty acid free (EFAF) BSA vs. Fraction V BSA ($P < 0.01$). There was, however, there was no temperature x BSA type interaction. Data are least-squares means ± SEM. Replications were 3 and number of oocytes per treatment were 57-58. Bottom panel. The effect of O_2 concentration (5% or 20.95%) during heat shock of two-cell embryos on subsequent development to the blastocyst stage. Results are the percentage of two-cell embryos that became blastocyst on Day 8 post-insemination. Percent blastocyst was affected by incubation temperature ($P < 0.01$) and O_2 concentration ($P = 0.07$). Data are least-squares means ± SEM. Replications were 4 and number of oocytes per treatment were 120-122.

($P = 0.07$) even though embryos were in the presence of low O_2 for only 12 h at the two-cell stage. However, culturing two-cell embryos in low O_2 tensions did not decrease the effect of heat shock on embryonic development as indicated by the lack of oxygen x temperature interaction. Heat shock reduced development from 53.5 to 27.0% at 20.95% O_2 and from 69.4 to 29.9% in 5% O_2.

Rectal Temperatures of Heat-Stressed Lactating Dairy Cows

The average peak air temperature and peak black globe temperature (Figure 2-6, top panel) on the days when the experiment was performed were 34.5° and 33.7°C, respectively. Mean rectal temperatures (Figure 2-6, bottom panel) ranged from a low of 38.6°C (0800 h) to a high of 40.5°C (1700 h). Mean respiration rates ranged from a low of 71.8 breaths per minute (000 h) to a high of 105 breaths per minute (1200 h; data not shown). Coat color affected ($P < 0.001$) rectal temperatures of cows (Figure 2-6, bottom panel), with predominantly white cows having lower rectal temperatures that predominantly black cows.

Exposure of Embryos to a Pattern of Temperatures Similar to Those Experienced by Heat-Stressed Cows

Results from the previous experiment were used to design a pattern of culture temperatures that would mimic the 24-h variation in body temperatures (Figure 2-8, top panel). Applying heat shock in this pattern during the first 24 h after insemination did not affect embryonic development to the blastocyst stage (Figure 2-8, bottom panel). In contrast, application of this pattern of fluctuating temperatures for 192 h (from d 0-8 after insemination) reduced embryonic development to the blastocyst stage ($P < 0.05$).

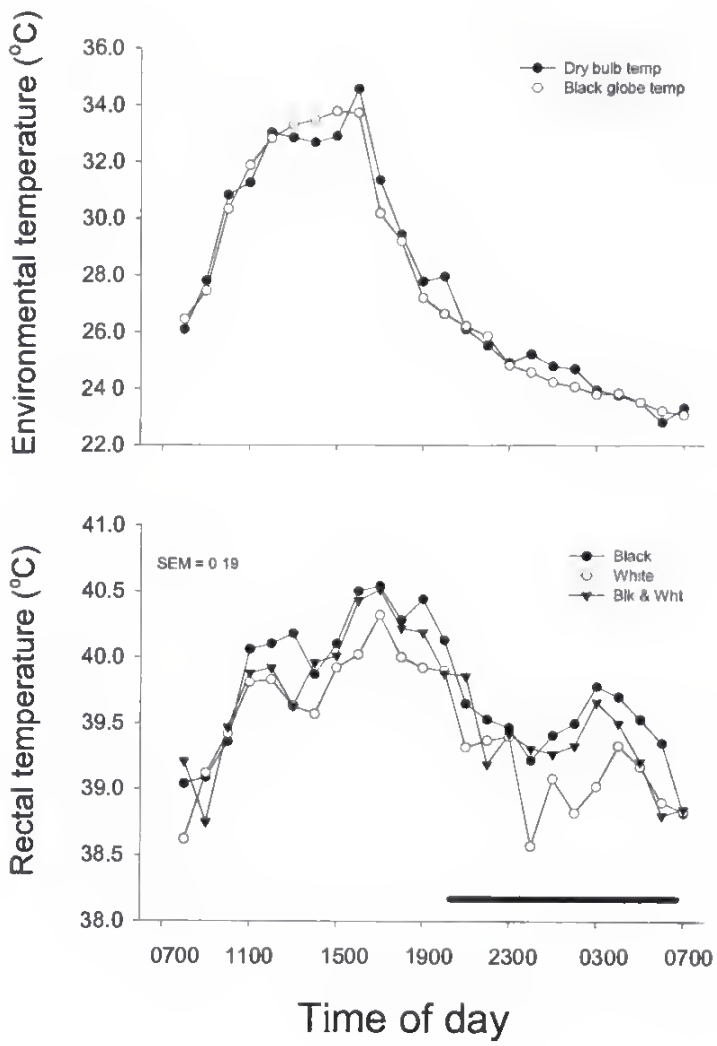


Figure 2-6. Rectal temperatures of lactating dairy cows under heat stress conditions in North Florida. The top panel shows dry bulb and black globe temperature while the bottom panel shows rectal temperatures for cows that are predominantly black (n=9), about equally black and white (n=8), and predominantly white (n=8). Rectal temperature was affected by coat color ($P < 0.001$). Data are least-squares means ± SEM. The black bar shows the period from sunset to sunrise.

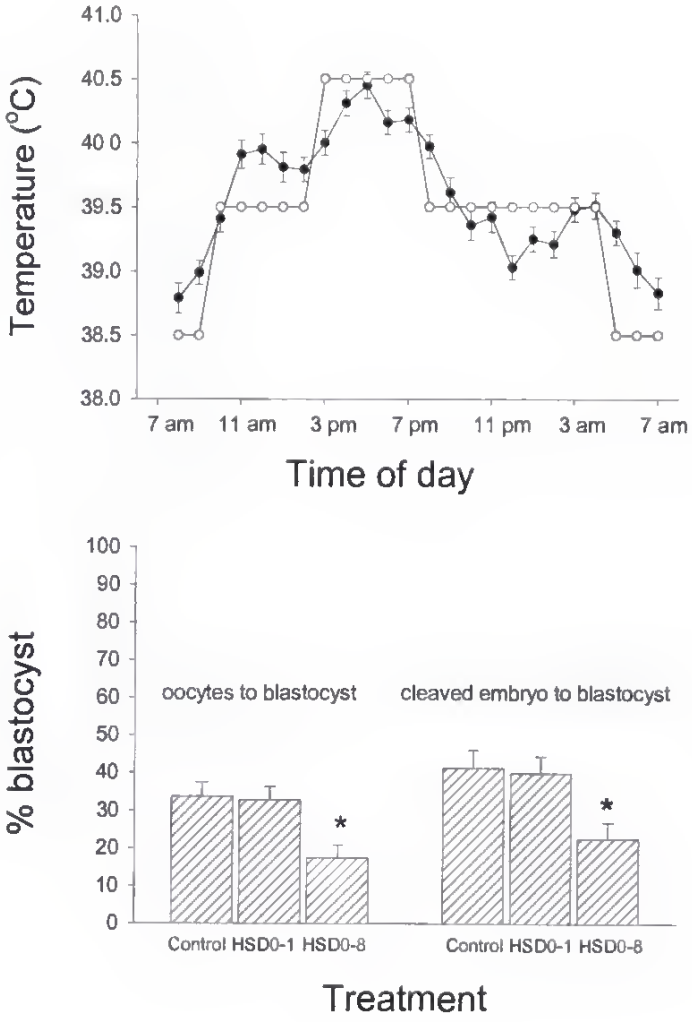


Figure 2-7. Effect of exposing cultured embryos to a pattern of temperatures similar to that experienced by heat stressed cows. Top panel: closed circles represent rectal temperatures of heat-stressed, lactating dairy cows in North Florida (least-squares ± SEM). Open circles represent culture temperatures for heat-shocked embryos. Bottom panel: Percent of oocytes and percent of cleaved embryos becoming blastocyst for embryos exposed to 38.5°C continuously (Control), heat shock for 24 h beginning at the end of fertilization (HSD0-1) or heat shock throughout the culture period (HSD0-8). Replications were 4 and number of oocytes per treatment were 121-122. * = different from control $P < 0.05$.

Discussion

The results of this study indicate that embryonic development can be disrupted by heat shock of 41.0°C applied during fertilization and at the one- and two-cell stage of development. Further, exposing early embryos to temperatures similar to those experienced by heat-stressed dairy cows can reduce development to the blastocyst stage. Thus, under certain circumstances, embryonic development is likely to be compromised by the direct actions of elevated temperature on the oocyte and embryo. The finding that short-term heat shock (≤ 12 h incubation) only affected development at very high temperatures (41.0°C) may mean that certain causes of heat-associated infertility associated with mild maternal hyperthermia is the result of effects other than direct actions of elevated temperature on embryonic survival.

Many previous reports have indicated deleterious effects of elevated temperature on oocytes and early embryos (Gwazdauskas et al, 1992; Ealy et al., 1995; Edwards and Hansen, 1997; Aréchiga and Hansen, 1998). In those experiments, however, temperature was confounded with changes in pH because CO_2 solubility in the medium decreases as temperature increases. Here it is shown that, even when controlling for pH changes, decreased development follows when either oocytes, one- or two-cell embryos are exposed to elevated temperature. Moreover, this effect of heat shock is not simply an artifact caused by high oxygen concentration in culture. Embryos are most commonly cultured in air, which has an O_2 content of about 20.95%, which is higher that what is found in the oviduct and/or the uterus (< 10%; 26). Given the fact that free radical formation can increase as a result of exposure to high O_2 tensions (Fowler and Callingham, 1978; Goto et al., 1993) and elevated temperatures (Flanagan et al., 1998), it

is possible that the detrimental effects of heat shock in cultured embryos involves free radical formation. In addition, heat shock of cultured embryos decreases glutathione, a free radical scavenger (Aréchiga et al., 1995) and adverse effects of heat on embryonic development can be minimized by various antioxidants (Ealy et al., 1992; Malayer et al., 1992; Aréchiga et al., 1994; Aréchiga et al., 1995). Accordingly, it was tested whether low O_2 tension would reduce the detrimental effect of heat on two-cell embryos, presumably by lowering free radical production on two-cell embryos. There was no temperature by O_2 interaction, suggesting that the deleterious effects of heat shock did not depend upon a high O_2 environment.

Several studies have demonstrated superior development of bovine embryos cultured at 5% O_2 versus in air (Dumoulin et al., 1995; Takahashi et al., 1996; Fujitani et al., 1997; Lim et al., 1999). In this study, a beneficial effect of low O_2 was seen when embryos received low O_2 for only 12 h at the two-cell stage. The modified media filters used here offer a practical method for controlling oxygen concentration in embryo culture to increase blastocyst yield.

The type of BSA used to supplement the culture medium also had an effect on development of embryos to the blastocyst stage. In particular, embryos developed better in EFAF-BSA than in Fraction V BSA. Despite their superior development, embryos in EFAF-BSA were not more resistant to heat shock. The superior development of embryos in EFAF-BSA could be due in part to the removal of fatty acids and other impurities from Fraction V BSA. The Fraction V-EFAF BSA difference may also have reflected simple batch-to-batch variation (McKieman and Bavister, 1992) rather than any purification process-related effect.

In the experiment in which rectal temperatures of cows was determined, cows that were mostly white had lower rectal temperatures than cows that were mostly black. This is in accordance with previous observations that white cows exposed to intense solar radiation had lower rectal and surface temperatures than black cows (Hansen, 1990). In addition, King et al. (1988) reported that predominantly-white cows bred in warm months had a shorter interval from calving to conception than did predominantly-black cows. In contrast, Godfrey and Hansen (1996) failed to observe any effect of coat color on reproduction of Holstein cows in the Caribbean.

As others have shown (Ulberg and Burfening, 1967; Lenz et al., 1983), heat shock during fertilization reduced not only cleavage rate but the ability of cleaved embryos to develop. Again, a high temperature (41.0°C) was necessary to achieve this effect. The fact that 41.0°C affected fertilization rates may have been due in part to effects on the oocyte (Lenz et al., 1983; Baumgartner and Chrisman, 1987; Edwards and Hansen, 1997; Putney et al., 1988; Rocha et al., 1998) or spermatozoa (Lenz et al., 1983; Chandolia et al., 1999b). Heat shock could also affect syngamy or the first cleavage division. These processes involve microtubule and microfilament assembly (Kim et al., 1997) and cytoskeletal elements collapse and aggregate following heat shock (Welch and Suhan, 1985). Embryos formed from fertilization at 41.0°C had reduced developmental potential, perhaps because damage to the cytoskeleton or other intracellular organelles persists. In addition, embryos formed via fertilization with heat-damaged spermatozoa had lower developmental potential (Ulberg and Burfening, 1967). Interestingly, exposure of oocytes and spermatozoa to 40.0°C during fertilization did not affect cleavage rate but did increase subsequent embryonic development. Perhaps, 40.0°C was too mild to

damage gametes or embryos and the increased thermal energy at 40.0°C increased embryonic metabolic rate.

The present data do confirm that heat shocks such as experienced by cells of heat-stressed cows can compromise embryonic development. In vivo, heat-stressed cows typically experience daily fluctuations in body temperature that persist for many days. Results from the last experiment indicated that exposure of embryos to fluctuating temperatures similar to rectal temperatures of cows experiencing heat stress could decrease embryonic development in vitro if occurring for several days. Thus, a longer, more chronic heat shock may reduce embryo survival through direct actions of elevated temperature on embryo. One important note is that the temperatures chosen to perform this experiment were averages of rectal temperatures of cows experiencing heat stress and that uterine temperatures are ~0.2°C higher than rectal temperatures (Gwazdauskas et al., 1973). Thus temperatures experienced by embryos can be higher than used here.

Despite the fact that heat shock can disrupt development, a striking finding was that embryonic development was resistant to disruption by heat shocks of 39.5-40.5° for up to 12 h. Thus, it is possible that infertility associated with mild hyperthermia (Dunlap and Vincent, 1971) involves rather more chronic effects of heat shock exposure on embryos or is the result of other changes in oocyte, reproductive tract, or embryonic function independent of direct effects of heat shock on embryos.

In conclusion, embryonic development can be disrupted by short-term (9 and 12 h) heat shock applied during fertilization and at the one- and two-cell stage but only when the heat shock is severe (≥ 41.0°C). Further, exposing embryos throughout the 8 days of in vitro culture to a pattern of temperatures similar to those experienced by heat stressed

lactating dairy cattle affects their development to the blastocyst stage. In addition, the effects of heat shock are not due to artifacts caused by change in pH or high oxygen concentration.

CHAPTER 3
ALTERATIONS IN ULTRASTRUCTURAL MORPHOLOGY OF TWO-CELL BOVINE EMBRYOS PRODUCED IN VITRO AND IN VIVO FOLLOWING A PHYSIOLOGICALLY-RELEVANT HEAT SHOCK

Introduction

Development of the cleavage-stage embryo is very susceptible to disruption by elevated temperatures (Alliston et al., 1965; Ulberg and Burfering, 1967; Edwards and Hansen, 1996; Edwards and Hansen, 1997; Chapter 2). Temperatures that have little effect on survival of most cells can be lethal to preimplantation embryos. For example, development of mouse embryos was inhibited by exposure to 39.0°C beginning at the one-cell stage (Gwazdauskas et al., 1992) and development of bovine two-cell embryos was decreased by exposure to 41.0°C for as little as 4.5 h (Krininger et al., 2002). In contrast, temperatures in this range have no effect on survival of other cells (Rattan, 1998). Hypersensitivity of the preimplantation embryo to elevated temperatures is a transient phenomenon: effects of heat shock on development of cultured bovine embryos are reduced as embryos advance in development past the two- to four-cell stage (Edwards and Hansen, 1997; Krininger et al., 2002). The susceptibility of the embryo to elevated temperature is likely one of the major causes for the decrease in fertility and increase in embryonic mortality caused by heat stress in cattle (Dunlap and Vincent, 1971; Ealy et al., 1993; Ryan et al., 1993), sheep (Dutt, 1963), rabbits (Shah, 1956), and mice (Ozawa et al., 2002).

The cellular changes caused by elevated temperature that lead to a block in embryonic development are not well understood. In other cells, heat shock can cause cytoskeletal rearrangement (Coss and Linnemans, 1996) changes in mitochondrial membrane potential and permeability (Funk et al., 1999), organelle disruption and re-localization (Welch and Suhan, 1985; Wang et al., 1998), increased membrane fluidity (Coss and Linnemans, 1996; Wang et al., 1998), and membrane blebbing (Jewel et al., 1982). Most work on effects of heat shock on cultured cells involves temperatures higher than those that comprise embryonic survival or are experienced by heat-stressed females. Whereas most studies on the effects of heat shock on mammalian cells have used temperatures of 43.0°-45.0°C, temperatures that would be lethal to most mammals, maternal hyperthermia of 40.0°-41.0°C compromises pregnancy rate in cattle (Dunlap and Vincent, 1971; Turner, 1982; Putney et al., 1988; Ealy et al., 1993). The hypersensitivity of the early preimplantation embryo to heat shock as compared to embryos at later stages (Edwards and Hansen, 1997; Krininger et al., 2002) and to other cells implies that heat shock might be causing different types of cellular pathologies than those caused by the severe heat shocks required to disrupt most cell types.

Experiments on effects of heat shock on bovine embryos have depended largely on the in vitro-produced embryo as a model. It is not known, however, if bovine embryos that develop in vivo respond to the deleterious effects of elevated temperatures similarly as for in-vitro produced embryos. Embryos produced in vitro differ in several ways from embryos that develop in utero. For example, they can exhibit retarded development rate (Holm et al., 2002), increased content of lipid (Crosier et al., 2001) and lower survivability after freezing (Enright et al., 2000). Thus, there is a need to verify

that the in vitro-produced embryo is an acceptable model for studying effects of heat shock on embryonic development by testing whether similar types of changes are induced by heat shock in embryos that were produced in vivo.

The principle objective of this study was to characterize ultrastructural changes in two-cell embryos caused by exposure to a physiologically-relevant heat shock. Another objective was to evaluate whether differences exist in the fine structure of two-cell embryos produced in vitro as compared to embryos produced in vivo and to test whether heat shock induced similar changes in both types of embryos.

Materials and Methods

Materials

All chemicals, except otherwise specified, were from Sigma (St. Louis, MO). Reagents for in vitro production of embryos were obtained as described elsewhere (Chapter 2). FSH was Folltropin-V from Vetrepharm Canada (London, Ontario). Gonadotropin releasing hormone (GnRH) was Cystorelin® (Merial, Iselin, NJ) purchased from Ag-Tech (Manhattan, KS). Percoll was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Penicillin-streptomycin and the culture media SP-TL, Hepes-TL, and IVF-TL were from Cell and Molecular Technologies (Lavallette, NJ) and were used to prepare SP-TALP, Hepes-TALP, and IVF-TALP (Parrish et al., 1986). Potassium simplex optimized medium (KSOM; formulation MR-020-D) was also from Cell and Molecular Technologies and was used to prepare KSOM-Bovine Embryo 1 (KSOM-BE1) by the addition of 3 mg/ml essentially fatty acid free Fraction V BSA, 2.5 μg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids

(Minimum Essential Medium - purchased from Sigma) as described elsewhere (Soto et al., 2003). Bovine steer serum was from Pel Freez Biologicals (Rogers, AK). The medium CR1aa was prepared as described (Rosenkrans et al., 1993) and modified by addition of 3 mg/ml essentially fatty acid free Fraction V BSA, 2.5 μg/ml gentamicin, and essential and non-essential amino acids. Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). The prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was Lutalyse® and was donated by Pfizer Animal Health (Kalamazoo, MO). Glutaraldehyde, osmium tetroxide, ethanol, acetone, EMbed-812, uranyl acetate, Reynolds' lead citrate, Butvar, and copper grids were from Electron Microscopy Sciences (Fort Washington, PA).

Production of Embryos

In Vitro. Procedures for oocyte maturation, fertilization, and embryo culture were as described previously (Chapter 2). Briefly, ovaries were collected at a local abattoir located at a travel distance of approximately 1.5 h from the laboratory. Oocytes obtained by slashing the ovary were matured for 21 h and then inseminated with a cocktail of Percoll-purified spermatozoa from three different Angus bulls; a different group of bulls was used for each replicate. At 18 to 20 h after insemination, putative zygotes were denuded of cumulus cells by suspending them in Hepes-TALP medium containing 1000 units/ml of hyaluronidase type IV and vortexing in a microcentrifuge tube. Presumptive zygotes were then placed in groups of ~30 in 50 μl microdrops of KSOM-BE1 or modified CR1aa. All cultures at 38.5°C were performed in an environment of 5% CO_2 in humidified air. The percent CO_2 was adjusted in the incubators used for heat shock treatments (7% and 8% CO_2 for 41.0 and 43.0°C, respectively) to ensure that concentration of dissolved CO_2 was similar between treatment and to maintain medium

pH at ~7.4 (Chapter 2). The temperature of all incubators was calibrated routinely to assure accuracy of treatments.

In Vivo. Estrous cycles of three Angus cows were synchronized by injections, intramuscular injections, of 100 μg GnRH on Day 0, 25 mg $PGF_{2\alpha}$, on Day 7, and 100 μg GnRH on Day 9. On Day 19, superovulation was induced by i.m. injections of decreasing doses of FSH each morning and afternoon for 4 days (2 injections of 40 mg each on the first day, 2 injections of 30 mg each on the second and third day, and two injections of 20 mg each on the last day). Cows received 25 and 15 mg $PGF_{2\alpha}$, intramuscularly, coincident with the 6^{th} and 7^{th} FSH injections, respectively. Approximately 24 h after the last FSH injection, each cow was inseminated with semen from a different Angus bull and insemination was repeated 12 h later. Cows were killed approximately 48 h after the first insemination and embryos immediately flushed from the oviducts. This study was conducted with the approval of the Institutional Animal Care and Use Committee of the University of Florida.

Inhibition of Blastocyst Development by Heat Shock at the Two-Cell Stage

In vitro-produced two-cell embryos were collected at ~28 h post-insemination and cultured in fresh microdrops of KSOM-BE1 or modified CR1aa at one of two temperatures; 38.5°C (i.e., homoeothermic body temperature of the cow) or 41.0°C (characteristic temperature for heat-stressed cows; Chapter 2). Embryos cultured at 41.0°C were returned to control temperature (i.e. 38.5°C) after 6 h. On Day 5 after insemination, the group of embryos cultured in modified CR1aa medium were supplemented with 10% FBS (no FBS was added to the KSOM-BE1 cultured embryos).

Development to the blastocyst stage was evaluated on Day 8 post-insemination. The experiment was replicated five times with a total of 60-70 two-cell embryos/treatment.

Effects of Heat Shock on the Ultrastructure of Two-Cell Embryos Produced In Vitro

Two-cell embryos were collected at ~28 h post-insemination, placed in fresh microdrops of KSOM-BE1 and cultured for 6 h at one of three temperatures; 38.5°C, 41.0°C, or 43.0°C (severe heat shock). Immediately after treatment, embryos were processed for electron microscopy. The experiment was replicated 4 times on different days. For statistical analysis, electron micrographs of 1 embryo (selected at random) per replicate per treatment were used to take measurements on cellular morphology.

Effects of Heat Shock on the Ultrastructure of Two-Cell Embryos Produced In Vivo and Treated Ex Vivo

Two-cell embryos were obtained from three superovulated Angus cows. Immediately after slaughter, the oviducts of each cow were flushed with 10 ml sterile and pre-warmed (~39.0°C) Hepes-TALP into a sterile Petri dish. Embryos (n=38) were immediately collected and separated by stage of development (one-cell [n=13], two-cell [n=24], and 4-cell [n=1]). Only embryos that were at the two-cell stage were used for the experiment. Two-cell embryos from each cow were divided randomly in two groups and cultured in microdrops of KSOM-BE1 at 38.5°C or 41.0°C. After 6 h, embryos were processed for electron microscopy. For statistical analysis, electron micrographs of 1-2 embryo per cow per treatment were used to take measurements on cellular morphology (n = 3 for 38.5°C and 5 for 41.0°C, respectively).

Electron Microscopy

Embryos were fixed in 2% (v/v) glutaraldehyde in phosphate-buffered saline (PBS; 0.01 M sodium phosphate and 0.15 M sodium chloride - pH 7.2) at 4.0°C overnight (8-10 h). To ease the handling of embryos during dehydration and embedding, embryos were washed in PBS, individually placed on a glass slide, and a drop of 3% (w/v) molten low gelling temperature agar (from Sigma) in PBS was placed on the embryo while observing through a dissecting microscope. Once the agar was hardened, the excess was cut-off to leave a square (~ 1mm^2) of agar containing the embedded embryo. While in agar, embryos were post-fixed in 1% (w/v) buffered O_SO_4, dehydrated in ascending concentrations of ethanol and en bloc stained [2% (w/v) uranyl acetate in 75% (v/v) ethanol] overnight at 4.0°C. After further dehydration to 100% ethanol followed by 100% acetone, embryos were infiltrated with EMbed-812, and ultra-thin sections (~75 nm thick) placed in Butvar covered, 75 mesh copper grids. Sections were stained with 2% (w/v) aqueous uranyl acetate and Reynolds' lead citrate before viewing with a Hitachi H-7000 Transmission Electron Microscope (Gaithersburg, MD). In addition, semi-thin sections (~500 nm) were collected for light microscopy examinations of whole embryos. Sections were placed on a glass slide and dried on a hot plate (~50.0°C). A drop of toluidine blue O stain [1:1 mixture of 0.2 (w/v)% toluidine blue and 2% (w/v) borax] was added to the sections and incubated on the hot plate for approximately 1 min (before the slide dried out). The sections were rinsed in a stream of distilled water prior to visualization.

Morphometric Analysis of Electron Micrographs

Electron micrographs of sections that contained the nucleus of at least one blastomere were used to take measurements on several aspects of cellular morphology. In addition, electron micrographs were assessed visually to determine effects of heat shock on other aspects of cellular morphology. For each embryo, morphometric analysis was performed using SIS Megaview III camera and software (Soft Imaging System, Lakewood, CO). The distance from the plasma membrane to the organelle-containing region of the cytoplasm was measured at 10 random locations from each of four pictures per embryo (i.e. 40 random measurements/embryo). The distance between the two membranes comprising the nuclear envelope was measured at five random locations around the nucleus in each of four pictures per embryo. The average of these measurements was used for statistical analysis. The percent of mitochondria exhibiting a swollen morphology was determined by counting the total number of mitochondria as well as the number of swollen mitochondria in each of four pictures per embryo.

Statistical Analysis

Effect of culture temperature on percent of two-cell embryos that developed to the blastocyst stage was analyzed by least-squares analysis of variance using the Proc GLM procedures of SAS (SAS for Windows, Version 8e, Cary, NC). Percent development was calculated for each replicate. Percentage data were analyzed before and after transformation by arcsine transformation. The analysis of transformed data was used to obtain probability values while analysis of untransformed data was used to obtain least-squares means ± SEM. Heat shock effects on the distance from the plasma membrane to the outermost organelles, proportion of mitochondria exhibiting a swollen morphology,

and the distance between the two membranes of the nuclear envelope was also analyzed by least squares analysis of variance using Proc GLM procedures. Except for distance between the two nuclear membranes, data exhibited heteroscasdicity. Accordingly, data were transformed by log transformation before analysis. The analysis of log-transformed data was used to obtain probability values while analysis of untransformed data was used to obtain means ± SEM.

Results

Inhibition of Embryonic Development by Heat Shock at the Two-Cell Stage

As shown in Figure 3-1, exposure of two-cell embryos to 41.0°C for 6 h reduced the percent of two-cell embryos that were blastocysts at Day 8 after fertilization ($p < 0.001$). The reduction in development occurred for both media and the temperature x medium interaction was not significant.

Heat-Shock Induced Changes in Ultrastructure of Two-Cell Embryos Produced In Vitro

The cytoplasm of embryos cultured at 38.5°C was occupied primarily by vesicles and mitochondria that extended from the perinuclear region to the edge of the blastomere (Figure 3-2A and 3-2B). Vesicles appeared to be, at least in part, Golgi. Indicative of their immature status, mitochondria displayed a hooded morphology and had few cristae. Embryos contained few endoplasmic reticulum but annulate lamellae (precursor organelles of the endoplasmic reticulum) were more evident. Lysosomes were also evident although in scarce abundance. Occasionally, a nucleolus (Figure 3-4A) was apparent in the nucleus. The plasma membrane of the blastomere was invested with

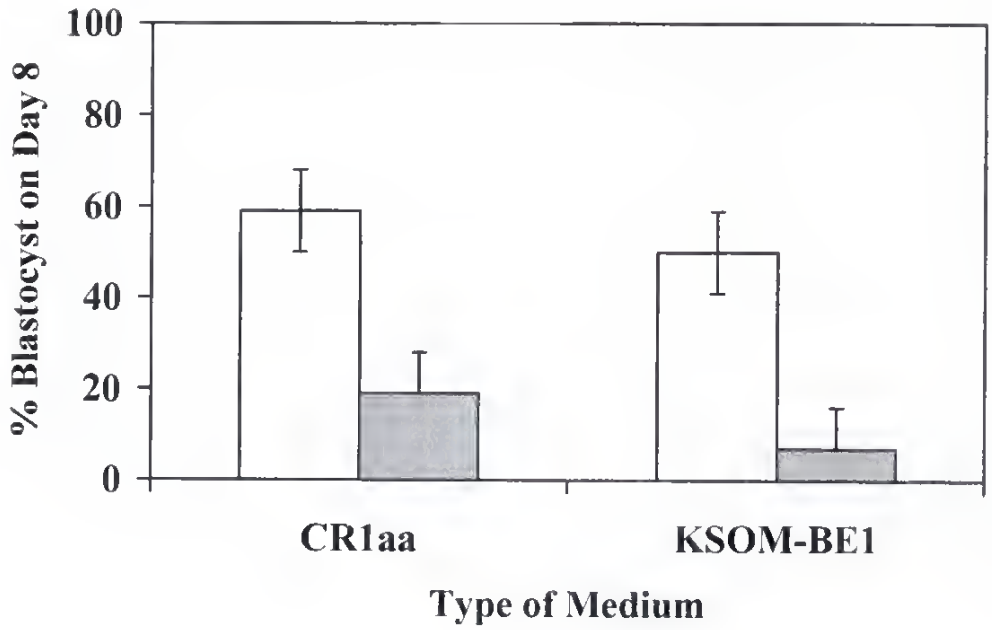


Figure 3-1. Inhibition of embryonic development by heat shock at the two-cell stage. Open bars represent control embryos while grey bars represent heat-shocked (6 h) embryos. Data represent least-squares means ± SEM of five replicates with a total of 60-70 two-cell embryos/treatment.

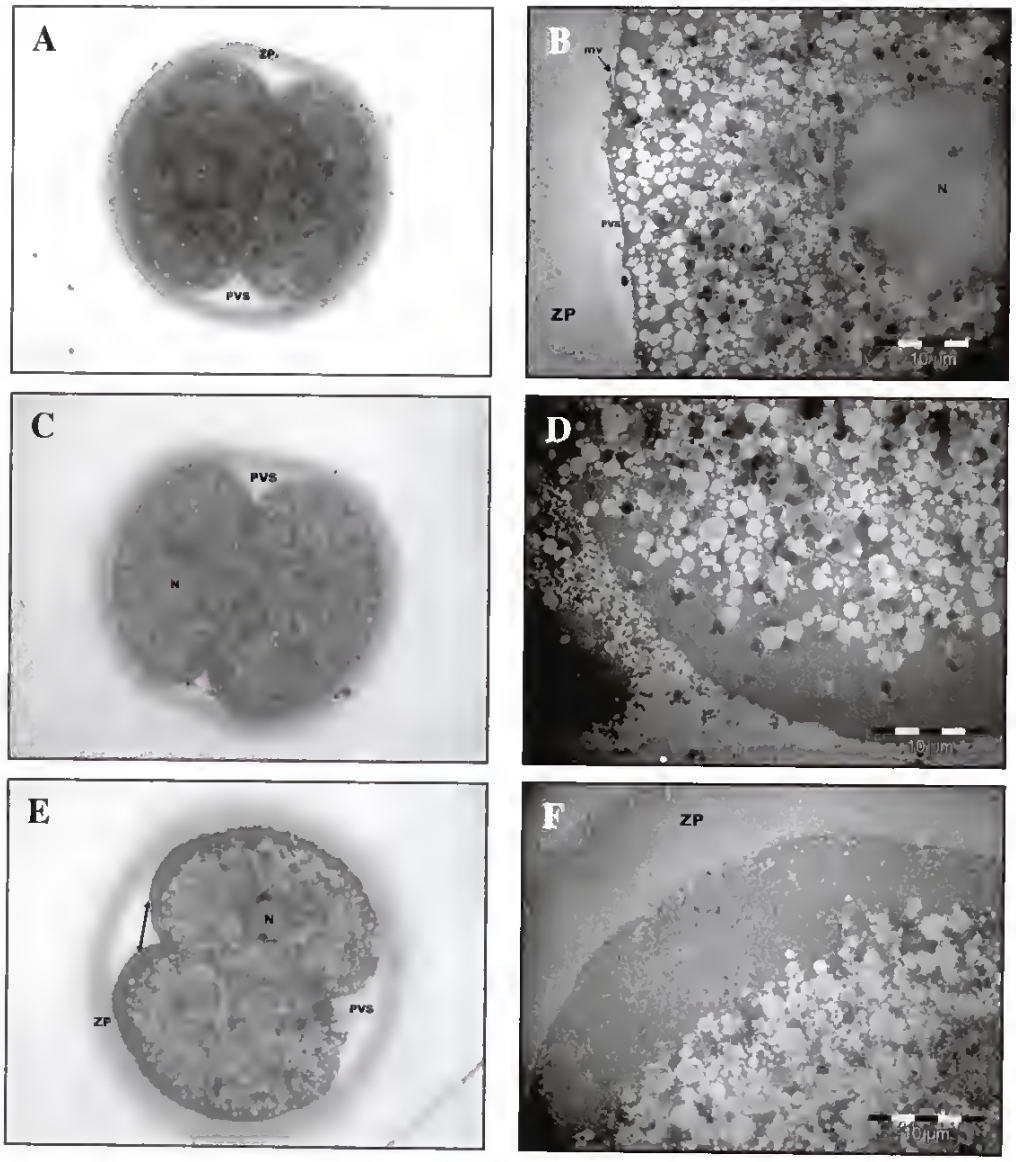


Figure 3-2. Effects of heat shock on morphology of two-cell embryos produced in vitro as determined by light microscopy (A, C, E) of 500 nm sections stained with toluidine blue O stain or by transmission electron microscopy (B, D, F). Embryos were cultured for 6 h at 38.5°C (A-B), 41.0°C (C-D), or 43.0°C (E-F). Arrows show area of the cytoplasm devoid of organelles. ZP = zona pellucida, PVS = peri-vitelline space, N = nucleus, mv = microvilli.

numerous microvilli and clathrin-coated pits could be observed occasionally (data not shown).

Upon heat shock, all organelles moved centrally to leave the periphery of the blastomere devoid of organelles (Figure 3-2C to 3-2F). The distance from the plasma membrane to the organelle-containing region of the cytoplasm was increased by exposure to 41.0° and 43.0°C (means ± SEM = 604 ± 132, 2261 ± 444, and 4463 ± 1915 nm for 38.5°, 41.0° and 43.0°C; $p<0.01$). In addition, numerous mitochondria were swollen in heat-shocked embryos. The percent of mitochondria showing a swollen morphology was increased ($p<0.001$; Figure 3-3) by both heat shocks (0 ± 0.25, 15 ± 4, and 74 ± 20% for 38.5°, 41.0°, and 43.0°C, respectively).

Heat shock at 43.0°C caused some ultrastructural changes that were not apparent in embryos exposed to 41.0°C. Heat shock at 43.0°C, but not at 41.0°C, increased ($p<0.001$) the distance between the two membranes comprising the envelope nuclear envelope (Figure 3-4F). Distance between the membranes was 45 ± 5, 51 ± 1, and 156 ± 5 nm for 38.5°C, 41.0°C, and 43.0°C, respectively. After culture at 43.0°C, the organelles were more tightly redistributed towards the perinuclear region and could no longer be recognized (Figure 3-2E and F and 3-6G to 3-6I). In addition, culture at 43.0°C resulted in vacuolization of the nucleoplasm (Figure 3-4C) and cytoplasm (Figure 3-2F and 3-5C). The cytoplasm of embryos cultured at 43.0°C for 6 h was covered with heterogeneous dense content (Figure 3-6G to 3-6I). The chromatin underwent severe degeneration and had a spotty appearance (Figure 3-4F). Moreover, embryos exhibited increased numbers of lysosomes (Figure 3-6H) and withdrawal of microvilli (data not shown).

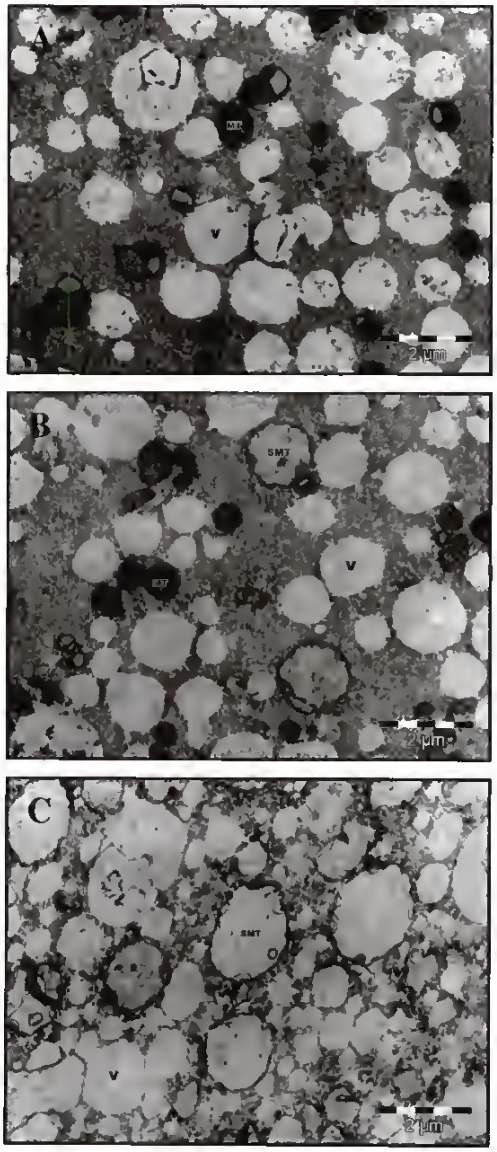


Figure 3-3. Heat-shock induced alterations in mitochondrial morphology. Two-cell embryos produced in vitro were cultured for 6 h at 38.5°C (A), 41.0°C (B), or 43.0°C (C). MT = mitochondrion, V = vesicle, SMT = swollen mitochondrion.

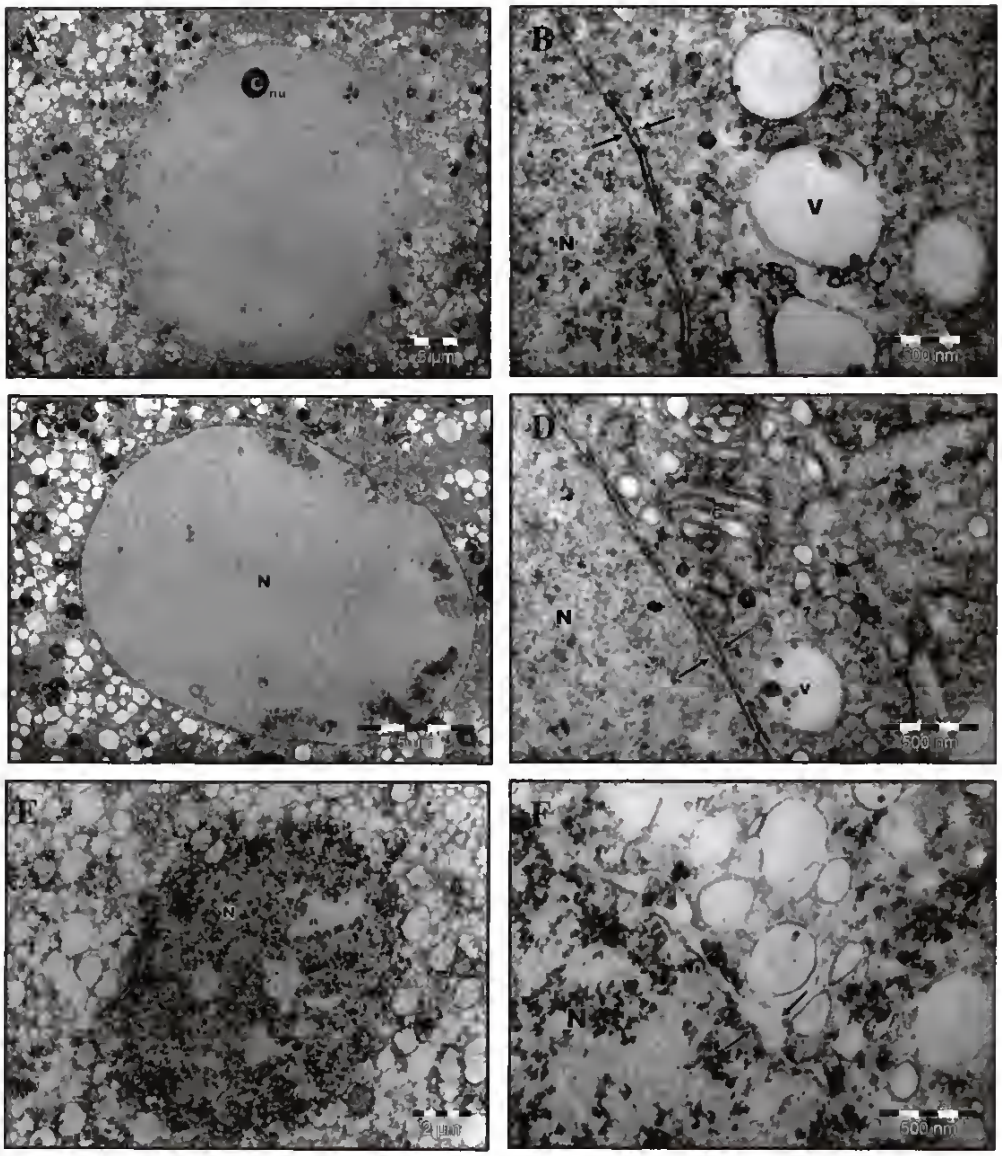


Figure 3-4. Effects of heat shock on nuclear morphology (A, C, E) and distance between the two membranes comprising the nuclear envelope (B, D, F). Two-cell embryos produced in vitro were cultured for 6 h at 38.5°C (A-B), 41.0°C (C-D), or 43.0°C (E-F). N = nucleus, nu = nucleolus, G = Golgi apparatus, V = vesicle; arrows point at the nuclear envelope.

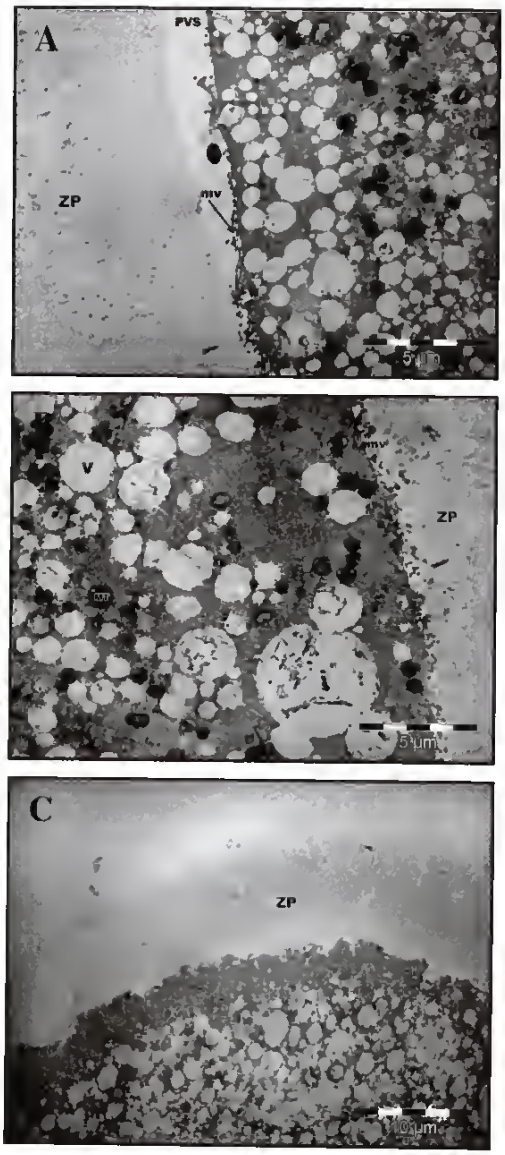


Figure 3-5. Disruption of plasma membrane by heat shock. Two-cell embryos produced in vitro were cultured for 6 h at 38.5°C (A), 41.0°C (B), or 43.0°C (C). mv = microvilli, ZP = zona pellucida, PVS = peri-vitelline space, V = vesicle, MT = mitochondrion.

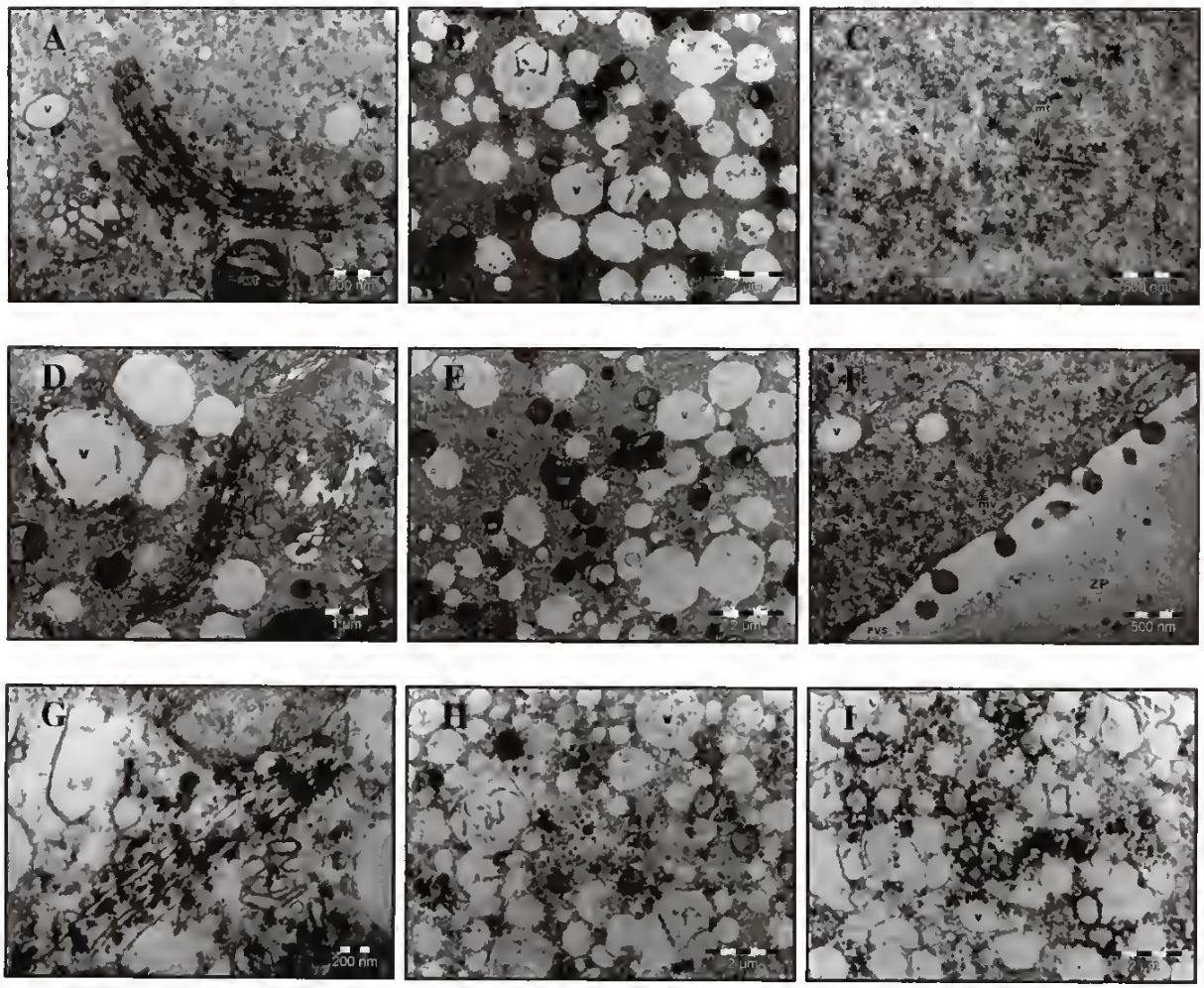


Figure 3-6. Representative micrographs demonstrating appearance of cytoplasmic components in two-cell embryos produced in vitro and cultured for 6 h at 38.5°C (A-C), 41.0°C (D-F), or 43.0°C (G-I). V = vesicle, Ly = lysosomes, ER = endoplasmic reticulum, SMT = swollen mitochondrion, AL = annulate lamellae, G = Golgi apparatus, MT = mitochondrion, V = vesicle, mv = microvilli, ZP = zona pellucida, PVS = peri-vitelline space, mt = microtubule.

Heat-Shock Induced Changes in Ultrastructure of Two-Cell Embryos Produced in Vivo and Treated Ex Vivo

Embryos recovered from superovulated cows and treated ex vivo were ultrastructurally comparable (Figures 3-7 and 3-8) to embryos produced in vitro (Figures 3-2 to 3-6). Like for embryos produced in vitro, the cytoplasm of embryos produced in vivo and cultured for 6 h was filled primarily with vesicles and mitochondria. Other organelles, including endoplasmic reticulum, Golgi apparatus, annulate lamellae, and lysosomes, were indistinguishable from in vitro embryos. Heat shock of 41.0°C for 6 h caused the same alterations in ultrastructural morphology as for in vitro embryos. In particular, the organelles of the embryos treated at 41.0°C for 6 h pulled away from the plasma membrane and towards the nucleus to leave an organelle-free area around the edge of the blastomere (Figure 3-7). The distance from the edge of the plasma membrane to the organelle-containing region of the cytoplasm was 743 ± 80 vs. 2553 ± 518 nm, for embryos at 38.5°C and 41.0°C, respectively ($p < 0.05$). The percent of mitochondria that exhibited a swollen morphology was also increased by heat shock (percent swollen = 0.7 ± 0.6 vs. 7 ± 2 for 38.5°C and 41.0°C, respectively; $p = 0.07$; Figure 3-8). A notable finding was the fact that lysosomes appeared with more frequency in the embryos produced in vivo and heat shocked at 41.0°C for 6 h than for embryos produced in vitro and treated under similar conditions (data not shown).

Discussion

The present study demonstrates that disruption of development caused by exposure of two-cell bovine embryos to a physiologically-relevant heat shock involves alterations to the cytoskeleton and mitochondria. The observation that there were no

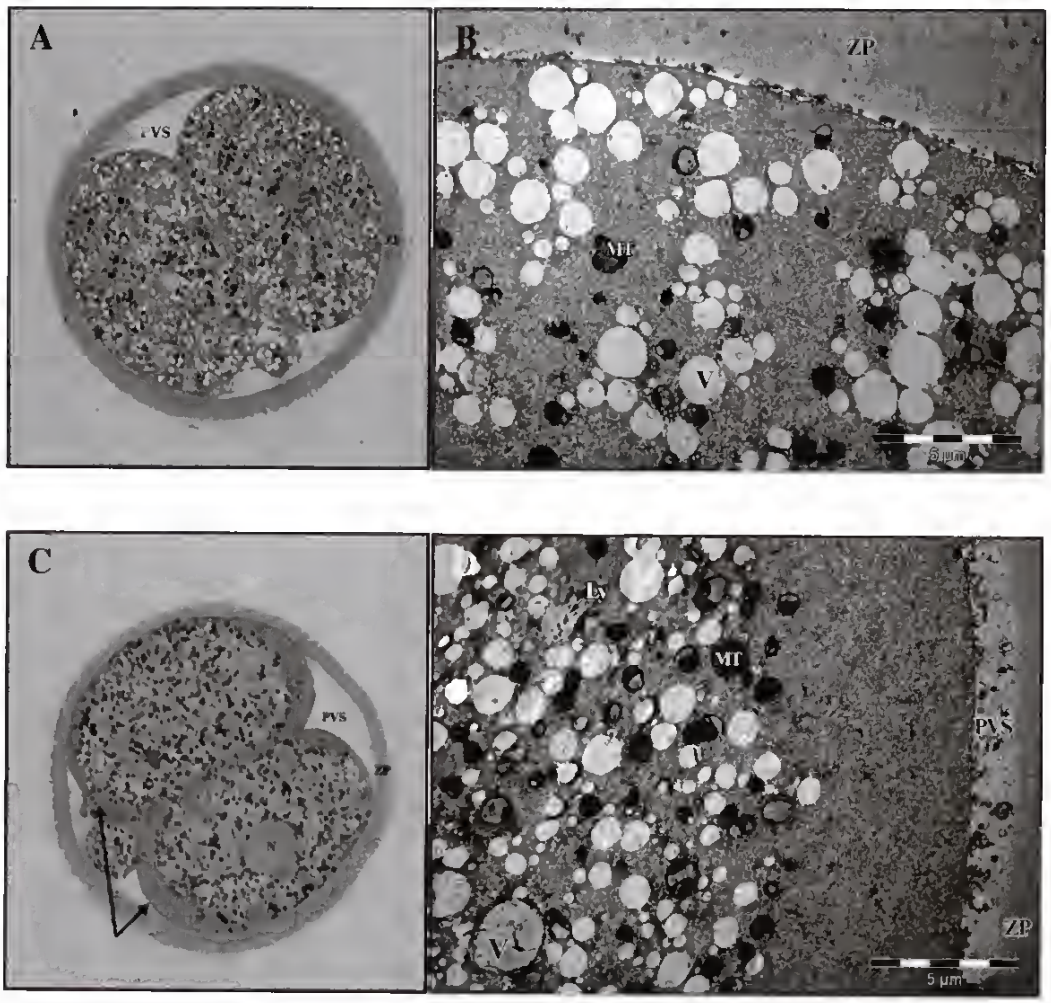


Figure 3-7. Effects of heat shock on morphology of two-cell embryos produced in vivo and treated ex-vivo as determined by light microscopy of 500 nm sections stained with toluidine blue O stain (A, C) or by transmission electron microscopy (B, D). Embryos were cultured for 6 h at 38.5°C (A-B) or 41.0°C (C-D). Arrows show area of the cytoplasm devoid of organelles. ZP = zona pellucida, PVS = peri-vitelline space, N = nucleus, MT = mitochondrion.

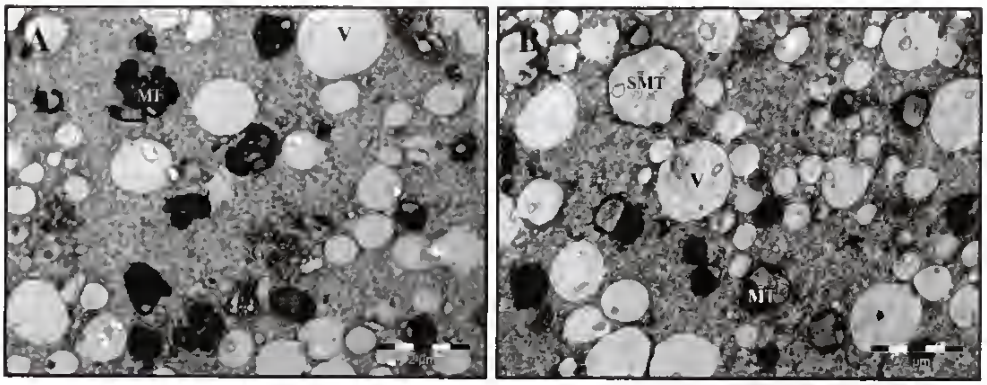


Figure 3-8. Heat-shock induced alterations in mitochondrial morphology of two-cell embryos produced in vivo. Embryos were cultured for 6 h at 38.5°C (A) or 41.0°C (B). MT = mitochondrion, V = vesicle, SMT = swollen mitochondrion, G = Golgi apparatus.

differences observed at the ultrastructural level between two-cell bovine embryos produced in vitro and in vivo at 38.5°C or in their response to hyperthermia indicates that use of embryos produced in vitro to study heat shock is relevant to the embryo that develops in vivo, at least with respect to ultrastructural changes.

Much is known about the cellular mechanisms whereby heat shock disrupts cell proliferation and differentiation. Heat shock can induce morphological alterations to the cytoskeleton (Coss and Linnemans, 1996) and changes to mitochondrial morphology, function, and potential (Welch and Suhan, 1985; Cole and Armour, 1988; Wang et al., 1998; Funk et al., 1999; Maccouillard-Poulletier de Gannes et al., 2000; Willis et al., 2000). In addition, heat shock causes disruption and redistribution of organelles (Welch and Suhan, 1985; Wang et al., 1998; Funk et al., 1999), damages the nucleus (Welch and Suhan, 1985; Gniadecki et al., 2001), induces protein aggregation (Wachsberger and Coss, 1993), and induces functional and ultrastructural changes in membranes (Jewel et al., 1982; Lepock et al., 1982; Funk et al., 1999). However, most of the work regarding cellular effects of heat shock used temperatures above 43.0°C and is not relevant to effects of much milder heat shocks affecting embryo survival. Temperatures at 43.0°C or above are lethal to many mammals and well above the body temperatures associated with hyperthermia-induced embryonic death in cattle (i.e. ≤ 41.0°C; Dunlap and Vincent, 1971; Putney et al., 1988; Ealy et al., 1993). As the first experiment of this paper indicates, development of the two-cell bovine embryo is very susceptible to disruption by mild heat shock (i.e. 41.0°C for 6 h). How such a mild heat shock affects the cellular and sub-cellular processes orchestrating early embryonic development has not been well understood and is the topic of this paper.

The most obvious alterations to the ultrastructure of two-cell embryos produced in vitro and in vivo caused by heat shock were the movement of organelles away from the plasma membrane and the increase in the percent of mitochondria that exhibited a swollen morphology. The translocation of organelles towards the center of the blastomere would suggest that heat shock affected the cytoskeleton because this system is involved in active transport of organelles and other components of the cell using both microtubule- and actin filament-based systems of transport (i.e. dynein, kinesin; Capco, 1996; Stebbings, 1996). The movement of organelles in the heat-shocked two-cell embryo is reminiscent of the translocation of melanosomes in the fish melanophore. Movement of these melanized organelles via aggregation towards the perinuclear region or dispersion to the periphery of the dorsal skin cells is responsible for rapid background adaptation. Both microtubules and microfilaments are involved during aggregation and dispersion of the melanosomes (Skold et al., 2002). It would be of interest to determine if the aggregation of organelles following heat shock also involved similar patterns of movement of organelles on microtubules and microfilaments.

While present evidence suggests that heat shock fractures or alters the cytoskeleton, the specific alterations involved are not known. The specific cytoskeletal element affected by heat depends on cell type (Coss and Linnemans, 1996) and, in all likelihood, temperature. Further studies need to be conducted to verify that the movement of organelles caused by heat shock is a result of cytoskeletal rearrangement and to determine which elements are involved in this response. An additional question that arises is whether sensitivity of the cytoskeleton to 41.0°C is unique to embryos or a general characteristic of mammalian cells.

It is likely that disruption of the cytoskeleton is a major cause of the inhibition of development caused by heat shock. The cytoskeleton is a cellular component that is common to the plasma membrane, nuclear membrane, and cytoplasmic organelles. The three components of the cytoskeleton (i.e., actin filaments, microtubules and intermediate filaments) work together to enhance structural integrity, impart cell shape, provide cell motility, position mRNA and protein (Capco, 1996; Gniadecki et al., 2001), and play an important role in cell signaling (Hang et al., 1999), mitosis and cytokinesis (Lodish et al., 2000). During the first three cleavage divisions, the mammalian embryo spends the majority of the cell cycle in S and M phases (Barnes and Eyestone, 1990). These cell-cycle stages are differentially affected by elevated temperatures. For instance, exposure of cells to heat shock during the S phase results in spontaneous premature chromosome condensation and micronuclear formation (Swanson et al., 1995). Heat shock during the M phase results in disassembly of the mitotic spindle, failure of cytokinesis, and polyploidy (Coss et al., 1982; Vidair et al., 1993). Experiments to test whether heat shock of two-cell embryos results in micronuclei formation and/or alterations in the ploidy of the blastomeres would provide further evidence for cytoskeleton-mediated damage of the developmental program.

The second major effect of heat shock observed for both in vitro-produced embryos and embryos produced in vivo was swelling of mitochondria. Mitochondrial swelling occurs under several different stressful situations such as calcium overload (Ichas and Mazat, 1998; Halestrap et al., 2002), oxidative stress (Lemasters, 1999; Halestrap et al., 2002), ischemia/reperfusion (Ooie et al., 2001), chronic ethanol exposure (Li et al., 2001), and hyperthermia (Cole and Armour, 1988). Swelling of the

mitochondrial matrix has been hypothesized to be a result of changes in the mitochondrial permeability transition caused by the opening of the high conductance permeability transition pore on the inner mitochondrial membrane and resulting in unselective solute fluxes (Ichas and Mazat, 1998). Under this condition, mitochondria can no longer maintain a proton gradient (Willis et al., 2000), aerobic ATP synthesis is arrested, a progressive unfolding of the inner mitochondrial cristae occurs, and several apoptosis initiator components such as cytochrome c and apoptosis inducing factor are released (Ichas and Mazat, 1998; Susin et al., 1998).

The estimated 15% loss in functional mitochondria caused by heat shock at the two-cell stage could cause a reduction in ATP synthesis as has been seen in other cell types (Calderwood, 1987). The consequences for reduced mitochondrial capacity may not immediately impact energy metabolism in the two-cell embryo since there is little metabolic activity until the 12- to 16-cell stage (Khurana and Niemann, 2000) when a shift from oxidative phosphorylation to glycolysis occurs (Thompson et al., 1996; Cummins, 2001). The loss of mitochondria at the two-cell stage could adversely affect later energy metabolism unless these are replaced by increased mitochondrial biogenesis. In most cell types, the release of cytochrome c and apoptosis inducing factor would trigger caspase activation and apoptosis. However, the two-cell bovine embryo is unable to undergo apoptosis after heat shock (Paula-Lopes and Hansen, 2002a) and the loss of mitochondria due to heat shock would not cause cell loss through apoptosis at this stage. The severe heat shock of 43.0°C caused a wider range of pathologies than 41.0°C. These included separation of the two membranes comprising the nuclear envelope, development of a rough appearance on the plasma membrane that indicates loss of microvilli, an

increase in the number of lysosomes, appearance of electron-dense content in the nucleus and cytoplasm, and vacuolation of the cytoplasm and nucleoplasm. Some of these changes, including loss of microvilli (Leyko and Bartosz, 1986), changes in cellular surface morphology and nuclear membrane (Marcocci and Mondovi, 1990), as well as concentration of organelles towards the nucleus (Coakley, 1987), have also been seen in cells exposed to temperatures of 43.0°C. The separation of nuclear envelope membranes and loss of microvilli could be the result of cytoskeletal rearrangement because the nuclear matrix is tightly fastened to the cytoskeletal scaffold (Gniadecki et al., 2001). Alternatively, a heat shock as severe as 43.0°C could affect the fluidity of membranes (Lepock, 1982). The electron-dense material seen in embryos exposed to 43.0°C for 6 h is aggregated protein caused by heat denaturation and exposure of hydrophobic domains (Lepock et al., 2001; Wachsberger and Coss, 1993). The increase in numbers of lysosomes in embryos exposed to 43.0°C implies that the blastomeres were undergoing autodigestion.

There were few differences in ultrastructure between two-cell embryos produced in vitro vs. those produced in vivo at either 38.5°C or 41.0°C. This similarity in ultrastructure agrees with results of another study comparing cleavage-stage embryos produced in vitro with those produced in vivo (Plante and King, 1994). Later in development, differences emerge in volume density of total mitochondria, vacuoles, and nuclei (Crosier et al., 2001), lipid content (Crosier et al., 2001; Rizos et al., 2002), degree of compaction (Plante and King, 1994), and intracellular communication (Boni et al., 1999). The one difference detected between embryos produced in vitro and in vivo was the appearance of lysosomes after heat shock. These appeared with more frequency in

the embryos produced in vivo than for embryos produced in vitro. Perhaps, there are subtle differences between these two types of embryos that either make the embryos produced in vivo more sensitive to the effects of heat shock (as indicated by increased number of lysosomes) or, perhaps, more able to recover from heat shock (because the increased number of lysosomes allow greater repair of the cell) Taken together, the overall similarity in response to heat shock is indicative that, at least at this early stage of development, information on effects of heat shock on embryos produced in vitro is relevant to understanding actions of heat shock on embryos produced in vivo. One question that remains to be answered is whether the changes in ultrastructural morphology caused by heat shock would also occur in embryos that were located in vivo at the time of heat shock since it is possible that the reproductive tract environment could mitigate some embryonic responses to heat shock.

In conclusion, exposure of two-cell bovine embryos to elevated temperatures can disrupt embryonic development by causing alterations in the nucleus, cytoplasm, mitochondria, and other organelles. Most importantly, some of these changes, particularly to the cytoskeleton and mitochondria, occur at temperatures characteristic of cows experiencing hyperthermia-induced infertility.

CHAPTER 4
POSSIBLE INVOLVEMENT OF FREE RADICALS AND MITOCHONDRIAL DAMAGE IN THE DELETERIOUS ACTIONS OF A PHYSIOLOGICALLY-RELEVANT HEAT SHOCK IN TWO-CELL BOVINE EMBRYOS

Introduction

Exposure of preimplantation embryos to elevated temperature disrupts continued development (Alliston et al., 1965; Elliot et al., 1968; Gwazdauskas et al., 1992; Chapter 2). Temperatures causing this effect represent a relatively-mild elevation over hyperthermic temperatures. Cattle embryos, for example, 41.0°C can block development compared to culture at 38.5°C (Chapter 2; Chapter 3). An elevation in culture temperature of only 2.0°C (from 37.0 to 39.0°C) reduced development of mouse embryos (Gwazdauskas et al., 1992). Since temperatures reducing development are within the range of temperatures experienced by heat-stressed females, it is likely that the disruption of embryonic development caused by heat shock is of physiological relevance to the reduction in fertility experienced by heat-stressed females (Elliot and Ulberg, 1971; Wattemann and Bazer, 1985; Putney et al., 1988; Ealy et al., 1993).

Little is known about the mechanisms by which mild elevations in temperature cause such catastrophic effects on the preimplantation embryo. Most of the literature dealing with effects of heat shock on cells uses much higher temperatures – commonly 43°- 45°C – and the molecular and cellular changes affected by these temperatures may not occur in embryos exposed to 41.0°C.

One possible action of heat shock is to increase free radical production by the embryo. Heat shock of cells has been shown to increase the flux of cellular free radicals (Flanagan et al. 1998) by uncoupling mitochondria, one of the main reactive oxygen species generator, as well as by activating enzymes which are involved in the generation of free radicals (Skibba et al., 1989). The major evidence that heat shock increases free radicals in embryos comes from the mouse, where heat shock has been shown to increase intracellular hydrogen peroxide production (Ozawa et al., 2002) and decrease intracellular content of the antioxidant glutathione (Aréchiga et al., 1995). In addition, mouse embryos (Aréchiga et al., 1995; Aréchiga and Hansen, 1998) and bovine blastocysts (Ealy et al., 1992) can be made more resistant to heat shock by addition of antioxidants in the culture medium. Induced thermotolerance in mouse embryos can be blocked by inhibition of glutathione synthesis (Aréchiga et al., 1995).

Exposure of bovine two-cell embryos to 41.0°C for 6 h resulted in a 15% increase in the number of mitochondria undergoing swelling (Chapter 3). Heat shock also been causes mitochondrial swelling in other cell types (Cole and Armour, 1988; Welch and Suhan, 1985; Funk et al. 1999). Mitochondrial swelling is a phenomenon which results from loss of membrane potential as a result of the opening of the high conductance permeability transition pores on the inner mitochondrial membrane (Halestrap et al., 2002). Increased calcium and generation of reactive oxygen species promote permeability transition pore opening, which in turn causes uncoupling of oxidative phosphorylation and prevents mitochondria from synthesizing ATP (Lemasters, 1999; Halestrap et al., 2002). In non-embryonic cells, temperatures in the range of 41.0°C can impair electron transport along the respiratory chain (Floridi et al., 1987) and thereby

decrease the amount of ATP and phosphorylation efficiency (Findly et al., 1983; Calderwood, 1987; Macouillard-Poulletier de Gannes et al., 1998). Oxygen consumption would be expected to decline in heat-shocked cells undergoing mitochondrial damage. In fact, the respiratory properties of microglial cells were compromised during and immediately after heat shock with an immediate drop in oxygen consumption (Macouillard-Poulletier de Gannes et al., 2000).

There were three objectives for the present study. The first was to evaluate the possible role of free radicals in disruption of embryonic development caused by a heat shock. This question was evaluated by determining whether the magnitude of the reduction in development caused by heat shock was reduced by lowering oxygen tension and by determining whether heat shock reduced embryonic content of glutathione, the major cytoplasmic antioxidant in the cell. The second objective was to determine whether heat shock altered oxygen consumption and ATP content of the embryo in a manner consistent with a change in blastomere function caused by increased mitochondrial damage. The third objective of this study was to determine the time point in development at which heat shock at the two-cell stage blocks development, i.e. whether heat shock causes an immediate inhibition of development or insted compromises the ability of the embryo to proceed through the eight-cell stage, the time when embryos begin major transcriptional activity (Memili and First, 2000).

Materials and Methods

In Vitro Production of Embryos

Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as described elsewhere (Soto and Hansen, 2003; Chapter 2;

Chapter 3). Briefly, ovaries were collected at a local abattoir located at a travel distance of approximately 1.5 h from the laboratory. Oocytes obtained by slashing the ovary were matured for approximately 21 h and then inseminated with a cocktail of Percoll-purified spermatozoa from three different Angus bulls; a different group of bulls was used for each replicate. At 12-18 h post-insemination (hpi), putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium containing 1000 units/ml hyaluronidase type IV (Sigma, St. Louis, MO) and vortexing in a microcentrifuge tube. Presumptive zygotes were then placed in groups of ~30 in 50 μl microdrops of KSOM-BE1 or KSOM-BE2.

Heat Shock Treatment

Two-cell embryos were collected at 28-30 hpi and cultured in fresh microdrops of KSOM-BE1 or KSOM-BE2 at one of two temperatures: 38.5°C (i.e., homeothermic body temperature of the cow) or 41.0°C (characteristic temperature for heat-stressed cows; Chapter 2). After 6 h, embryos were either collected and analysed for oxygen consumption, glutathione content or ATP content, or returned to control temperature (38.5°C) until Day 3 or 8 post-insemination when subsequent development was assessed. All cultures at 38.5°C were performed in an environment of 5% CO_2 in humidified air except otherwise specified. The percent CO_2 was adjusted to 7% in the incubator used for heat shock to maintain medium pH similar to that of the control incubator (~7.4; Chapter 2). The temperature of all incubators was calibrated routinely to assure accuracy of treatments.

Effects of Oxygen Tension on Inhibition of Development Caused by Heat Shock

This experiment was performed to determine if culture in a low oxygen environment (5%) would reduce effects of heat shock on development as compared to culture in a air (20.95% oxygen). The design was a 2 x 2 factorial in which two temperatures (38.5 and 41.0°C) and two oxygen tensions (5 and 20.95%) were tested. Embryos were cultured in either high or low oxygen environment for the duration of the experiment beginning after removal from fertilization drops. For cultures under low oxygen environment, the gaseous phase was 5% CO_2, 5% O_2, and 90% N_2 for the control incubator and 7% CO_2, 5% O_2, and 88% N_2 for the heat shock incubator. Approximately 30 hpi, two-cell embryos were placed in fresh microdrops of KSOM-BE2 and cultured for 9 h at one of two temperatures in high or low oxygen tension. Embryos cultured at 41.0°C were returned to control temperature (i.e. 38.5°C) after 9 h. Development to the blastocyst stage was evaluated on Day 8 post-insemination. The experiment was replicated four times with a total of 53-77 two-cell embryos/treatment.

Effects of Heat Shock on Glutathione Content

Glutathione Recycling Assay. Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. Immediately after the 6 h culture, groups of 16-20 two-cell heat-shocked or control embryos were suspended in 5 µl KSOM-BE1 and stored at –20.0°C until analyzed for glutathione content. Glutathione content of embryos was determined as previously described (Aréchiga et al, 1995; Paula-Lopes et al., 2003a) by the use of the glutathione-recycling assay (Baker et al., 1990). This method is based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid; DNTB) to 2-nitro-5-thiobenzoic acid (a yellow

colored product) by reduced glutathione and the recycling of glutathione disulfide to reduced glutathione by glutathione reductase. On the day of the assay, groups of embryos were suspended in 45 µl deionized water and pipeted into separate wells of a 96-well microtiter plate. In addition to the embryo samples, 50 µl of standard (0 – 100 pmol glutathione per well) were pipeted into duplicate wells. This was immediately followed by addition of 100 µl reaction mixture (prepared by mixing 5.75 ml of 100 mM $NaPO_4$ buffer with 1 mM EDTA, pH 7.5, 5 ml of 1 mM DNTB, 5 ml of 1 mM NADPH, and 0.1 ml 200 U/ml glutathione reductase dissolved in $NaPO_4$–EDTA buffer) to the embryo samples or standards (all chemicals for the assay were from Sigma-Aldrich). Absorbance was read at 405 nm after ~6-8 min incubation using a microtiter plate reader (model EL 309; BIO-TEK Instruments Inc). The experiment was replicated on seven different days so that 7 pools of embryos (16-20 embryos/pool) were assayed for each treatment.

Effects of Heat Shock on Oxygen Consumption

Electrode preparation. All chemicals were obtained from Sigma-Aldrich at the highest available purity level unless otherwise stated. Solutions were made with deionized water from a Milli-Q Plus system water purifier (Millipore Co., Bedford, MA). The epoxy Epon 828 with m-phenylenediamine as the curing agent was purchased from Miller-Stephenson (Dandury, CT). Oxygen sensing electrodes were manufactured following a previously-described protocol (Jung et al., 1999). Briefly, a 1.5 cm by 25 µm-diameter platinum wire was attached to a 27-gauge syringe needle using silver epoxy (Epoxy Technology, Billerica, MA). The needles were baked for 1 h at 100°C. After baking, the platinum wire was immersed in a solution of 3 M KCN and 1 M KOH while a 3.4 V square wave at a frequency of 250 Hz was applied for 6-8 minutes to etch the

platinum wire to 1-3 μm diameter. The etched platinum wire and needle were then placed inside a pulled glass capillary that had been cut at the end such that the wire protruded from the tip. Next, the electrodes were dipped into epoxy (Epon 828 with m-phenylenediamine as the curing agent) and were allowed to dry overnight followed by curing at 100˚C for 2 h and then at 150˚C for an additional 2 h. After curing, a second etching procedure was undertaken under the same conditions as described above, with the exception that KOH was not present, to recess the electrode. The second etching step proceeded until the platinum wire was recessed 2-3 μm into the end of the glass pipette. The electrodes were then dipped into 7% (w/w) cellulose acetate in tetrahydrofuran for 60 sec. The final diameter of the electrodes was 2-5 μm.

All electrodes were tested and calibrated by addition of N_2, air, and O_2 to a solution at the electrodes. These solutions corresponded to partial pressures of oxygen of 0, 150 and 720 mm Hg, respectively. Testing was performed with the electrodes immersed in Krebs' Ringer buffer (KRB) which consisted of 118 mM NaCl, 5.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 20 mM Hepes at pH 7.4.

Oxygen consumption measurements. Embryos were collected at the two-cell stage at approximately 28 hours post-insemination (hpi), placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. Immediately after the 6 h culture, two-cell embryos were transferred to fresh KSOM-BE1 medium and transported in a portable incubator (Minitube, Verona, WI) set at 39.0°C. Embryos were then incubated at 38.5˚C in 5% CO_2 on a poly-l-lysine coated Petri dish for 20-30 min in KSOM-BE1. The Petri dish was transferred to a micro-incubator on the stage of a Zeiss Axiovert 35 microscope where the KRB was maintained at 37.0˚C. Stabilization of the electrodes was performed

by positioning the electrodes 100-200 μm away from the embryo. Once a stable baseline was observed for 2-5 min, the electrode was positioned 10-20 μm away from the embryo and measurements taken. The electrode was then inserted into the perivitelline space of the embryo and measurements recorded for 2 min. The electrode was repositioned outside the embryo (10-20 μm) and the measurement process repeated at least 3 times on each embryo (i.e., electrode was inserted at least three times through the zona).

Oxygen measurements were performed by applying a potential with a battery to a saturated sodium calomel electrode and using a Keithley 428 current amplifier to measure the current from the working electrode. The potential at the working electrode was -0.6 V vs. SSCE. The data were collected via a data acquisition board (Axon Digidata 1200) from Axon Instruments, Inc. (Foster City, CA) and a personal computer using Axoscope 8.1 software. Data were collected at 100 Hz and low pass filtered at 20 Hz. The oxygen concentration was determined by using the calibration curve. Oxygen consumption was calculated for each embryo as the difference in partial pressure of oxygen between measurements taken inside and outside the zona pellucida. These values were converted to mol/sec using an equation described by Mueller-Klieser (1984) and assuming diameter of the embryo was 120 μm. The experiment was replicated three times with a total of 7-8 two-cell embryos/treatment.

Effects of Heat Shock on ATP Content

ATP Assay. Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. The ATP assay was performed on pooled groups of 3-5 two-cell embryos per treatment immediately after the 6-hr culture period. The ATP determination kit, from Molecular

Probes (Eugene, OR), is a bioluminescence assay based on the requirement of firefly luciferase for ATP to produce light. The standard reaction solution was prepared following the manufacturer's specifications. A series of freshly prepared ATP standards (0.08 to 5 pmol /tube dissolved in distilled water) were used to generate a standard curve. The assay consisted of addition of a 10 µl embryos (8µl distilled water plus ~2 µl embryos and culture medium) or 10 µl standards to 100 µl reaction mixture. Luminescence was detected with the use of a luminometer (AutoLumat LB953, Wallac Inc., Gaithersburg, MD). Reaction mixture was added automatically by the luminometer immediately prior to measurement and each sample was measured for 5 min. The experiment was replicated on eight different days with a total of 21 pools (38.5°C) or 24 pools (41.0°C) of 3-5 two-cell embryos.

Effects of Heat Shock at the Two-Cell Stage on Subsequent Development to Day Three Post-Insemination

Embryos were collected at the two-cell stage at approximately 28 hpi, placed in fresh drops of KSOM-BE1, and cultured at 38.5 or 41.0°C for 6 h. Culture dishes containing heat-shocked embryos were returned to control temperature until 72 hpi. Embryos were fixed in 4% paraformaldehyde (purchased as 8% solution from Electron Microscopy Sciences, Fort Washington, PA) in 0.2 M $NaPO_4$, pH 7.4 containing 0.15 M NaCl (phosphate-buffered saline; PBS) for 1 h. Nuclei of embryos were then stained with a 1-µg/ml solution of Hoechst 33342 dye in PBS supplemented with 1 mg/ml polyvinyl pyrrolidone (PBS+PVP) for 45 min. Embryos were washed three times in PBS+PVP prior to placing on glass slides containing 5 µl ProLong antifade solution (Molecular Probes, Eugene, OR). Total and condensed/pycnotic nuclei were counted by viewing with a Zeiss Axioplan2 epifluorescent microscope (Carl Zeiss, Inc., Gottingen,

Germany). Images were acquired using AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Carl Zeiss, Inc., Thorwood, NY). The experiment was replicated on four different days with 46 embryos/treatment.

Statistical Analysis

Data were analysed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 8e, Cary, NC). In each case, replicate was considered as a random effect and other variables were considered as fixed effects. Tests of significance were made using error terms derived from calculation of expected means squares. For the experiment to test effect of oxygen tension on response to heat shock, data subjected to analysis were the percent of two-cell embryos that developed to the blastocyst stage calculated for each replicate. The mathematical model included main effects of temperature, oxygen tension, replicate, and their interactions. Percentage data were analysed before and after transformation by arcsine transformation. Analysis of transformed data was used to obtain probability values while analysis of untransformed data was used to obtain least-squares means ± SEM. For the ATP, glutathione and oxygen consumption experiments, the mathematical model included effects of temperature, replicate and temperature x replicate. For the experiment regarding effects of heat shock on development on Day 3 after insemination, percentage data were calculated for each replicate and untransformed and arcsine-transformed data were analysed using a mathematical model that included effects of temperature, replicate and temperature x replicate.

Results

Effects of Oxygen Tension on Inhibition of Development Caused by Heat Shock

Incubation of two-cell embryos at 41.0°C for 9 h reduced ($p = 0.06$) the proportion of embryos that became blastocysts at Day 8 after insemination (Figure 4-1). The reduction in development occurred for embryos cultured under both low and high oxygen environments. There was a tendency ($p = 0.08$) for a greater proportion of embryos to form blastocysts under low oxygen tension than embryos cultured under high oxygen environment. There was a temperature x oxygen tension interaction ($p < 0.03$) that reflects the numerically greater reduction in development caused by heat shock for embryos under low oxygen tension.

Effect of Heat Shock on Glutathione Content

There were no differences in glutathione content (Figure 4-2) between control and heat-shocked embryos (3.42 ± 0.42 vs. 3.12 ± 0.42 pmol/embryo for embryos cultured at 38.5°C and 41.0°C, respectively).

Effect of Heat Shock on ATP Content

Heat shock did not decrease embryonic content of ATP (Figure 4-3). Values were 0.26 ± 0.06 pmol/embryo for embryos cultured at 38.5°C and 0.30 ± 0.05 pmol/embryo for embryos at 41.0°C.

Effect of Heat Shock on Oxygen Consumption

Representative photomicrographs illustrating the placement of the electrode inside and outside the perivitelline space are shown in Figure 4-4A while a representative pattern of changes in oxygen concentration as the electrode is moved from outside the

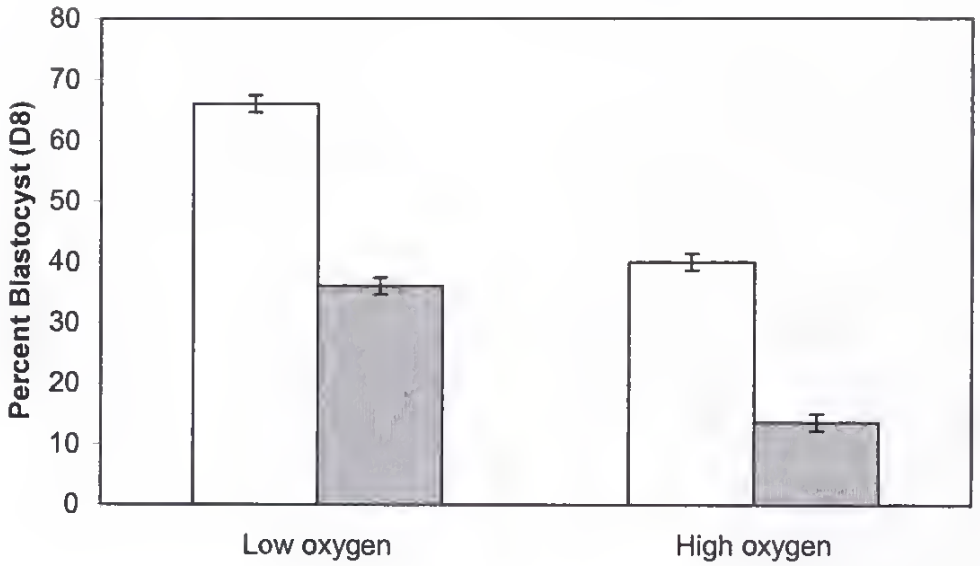


Figure 4-1. Effects of oxygen environment on responses of two-cell embryos to heat shock. Data represent the proportion of two-cell embryos that became blastocysts at Day 8 after insemination for embryos cultured in air (i.e., 20.95% oxygen) or under a low oxygen (5%) environment. Open bars represent control embryos while grey bars represent heat-shocked embryos. Values are least-squares means ± SEM of data from four replicates with 53-77 two-cell embryos per treatment.

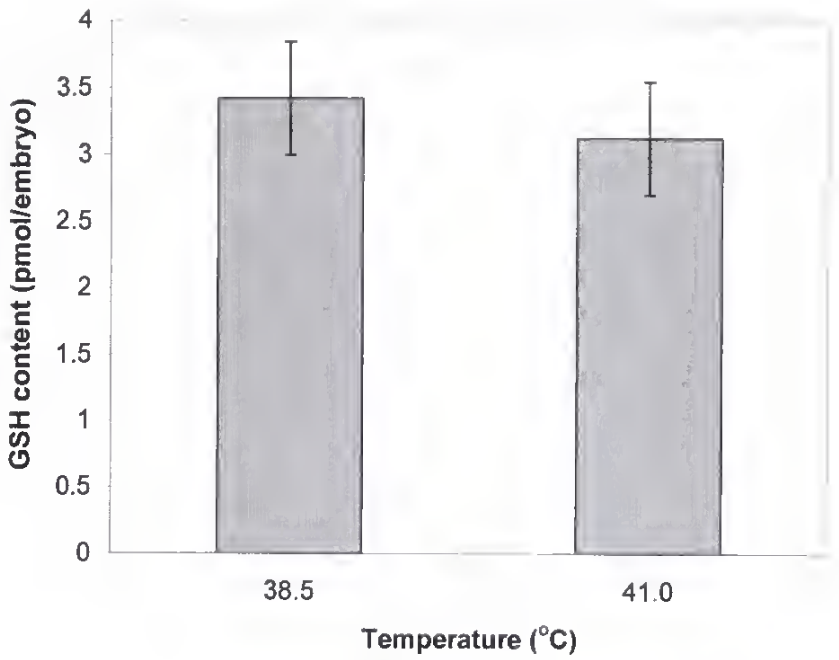


Figure 4-2. Effects of heat shock on glutathione content in two-cell bovine embryos. Embryos were heat-shocked or left as control at the two-cell stage, frozen immediately after treatment was finished, and analyzed on a later day for glutathione content. Values represent lsmeans ± SEM of seven replicates with 136 two-cell embryos per treatment.

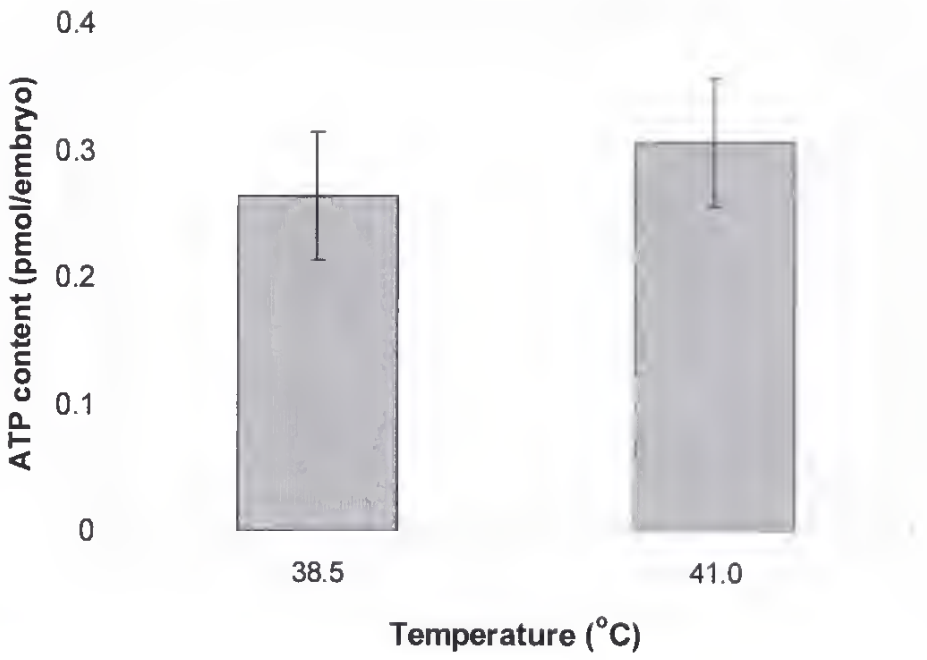


Figure 4-3. Effects of heat shock on ATP content in two-cell bovine embryos. ATP content of two-cell embryos (bottom panel) was determined immediately after 6 h treatment. Values represent lsmeans ± SEM of eight replicates with 105-122 two-cell embryos per treatment.

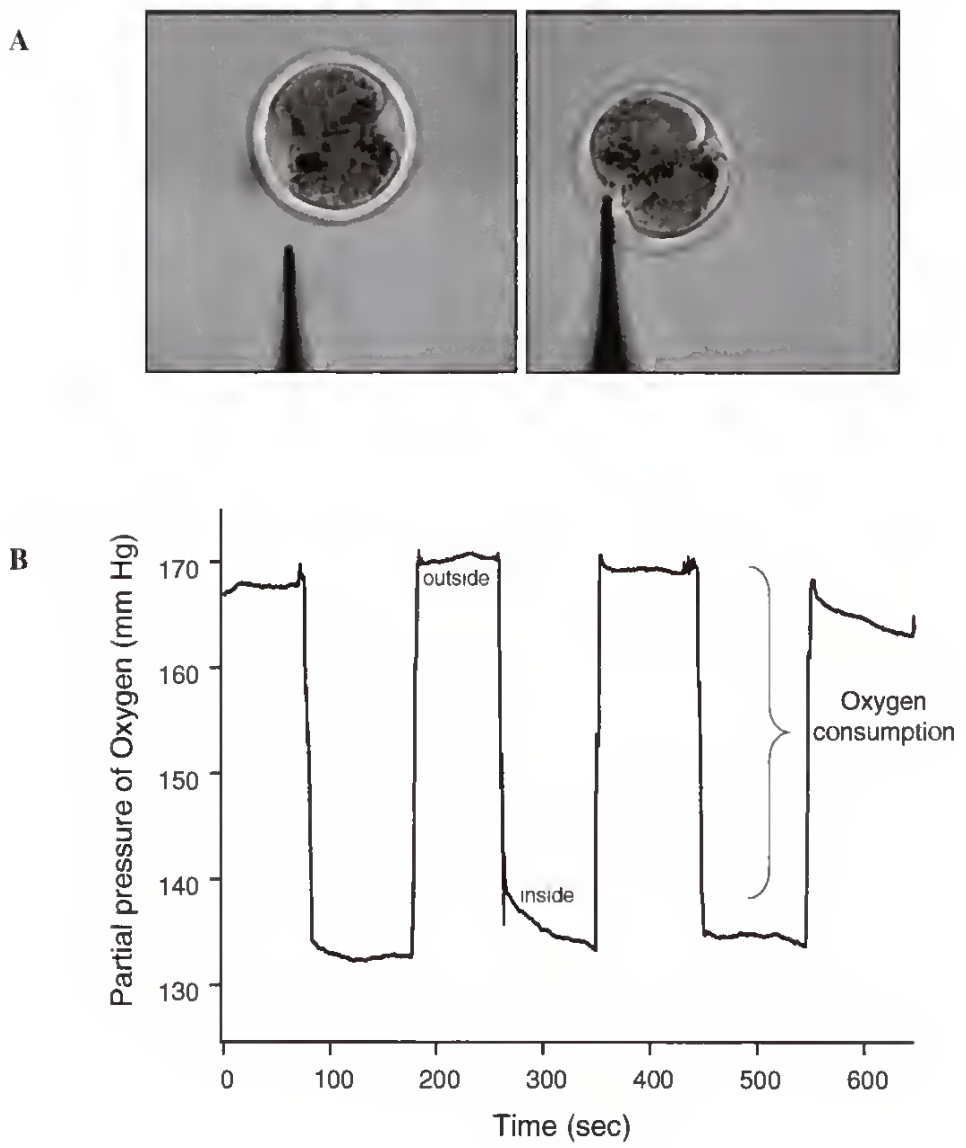


Figure 4-4. Method for measuring oxygen consumption of two-cell bovine embryos. Panel A are photomicrographs that illustrate the placement of oxygen electrodes outside and in close proximity to the zona pellucida (left) or inside the perivitelline space (right). Panel B shows a typical pattern of changes in oxygen concentration as the electrode is moved from outside the zona pellucida to inside the perivitelline space.

zona pellucida to inside the perivitelline space is shown in Figure 4-4B. No effect of heat shock on concentrations of oxygen outside or inside the zona pellucida or on oxygen consumption (calculated as the difference between oxygen concentrations inside and outside the zona pellucida) was observed. Least-squares means for oxygen consumption were 25.58 ± 5.4 mmHg (~$2.8x10^{-13}$ mol/sec) and 12.25 ± 5.12 mmHg ($1.3x10^{-13}$ mol/sec) for 38.5 and 41.0°C, respectively ($p = .18$).

Effects of Heat Shock at the Two-Cell Stage on Subsequent Development to Day Three Post-Insemination

Heat shock at the two-cell stage reduced the number of total nuclei from 10.3 ± 0.6 to 7.6 ± 0.6 ($p < 0.05$; Figures 4-5 and 4-6A). Heat shock tended to increase ($p = 0.08$) the number of pycnotic nuclei (Figure 4-6A; 0.43 ± 0.12 and 1.12 ± 0.12 for 38.5 and 41.0°C, respectively) and increased ($p < 0.01$) the percent of nuclei that were pyknotic (4.5 ± 4.6 vs. 21.8 ± 4.5% condensed for 38.5 and 41.0°C, respectively).

The stage of development at 72 h after insemination was evaluated to determine whether embryos subjected to heat shock at the two-cell stage became blocked in development at a specific stage (Figure 4-6B and 4-6C). The distribution of cell number at 72 hpi is shown in Figure 4-5B. It is apparent that a proportion of the heat-shocked embryos became blocked in development at the eight-cell stage. Only 25 ± 7% of the heat-shocked embryos developed past the 8-cell stage versus 67 ± 7% of control embryos ($p<0.05$). That the block to development occurred at this stage and not earlier was evident by the fact that heat shock did not affect the proportion of embryos developing past the two-cell stage (100 ± 0 vs. 98 ± 15%) or 4-cell stage (98 ± 15 vs. 96 ± 21%).

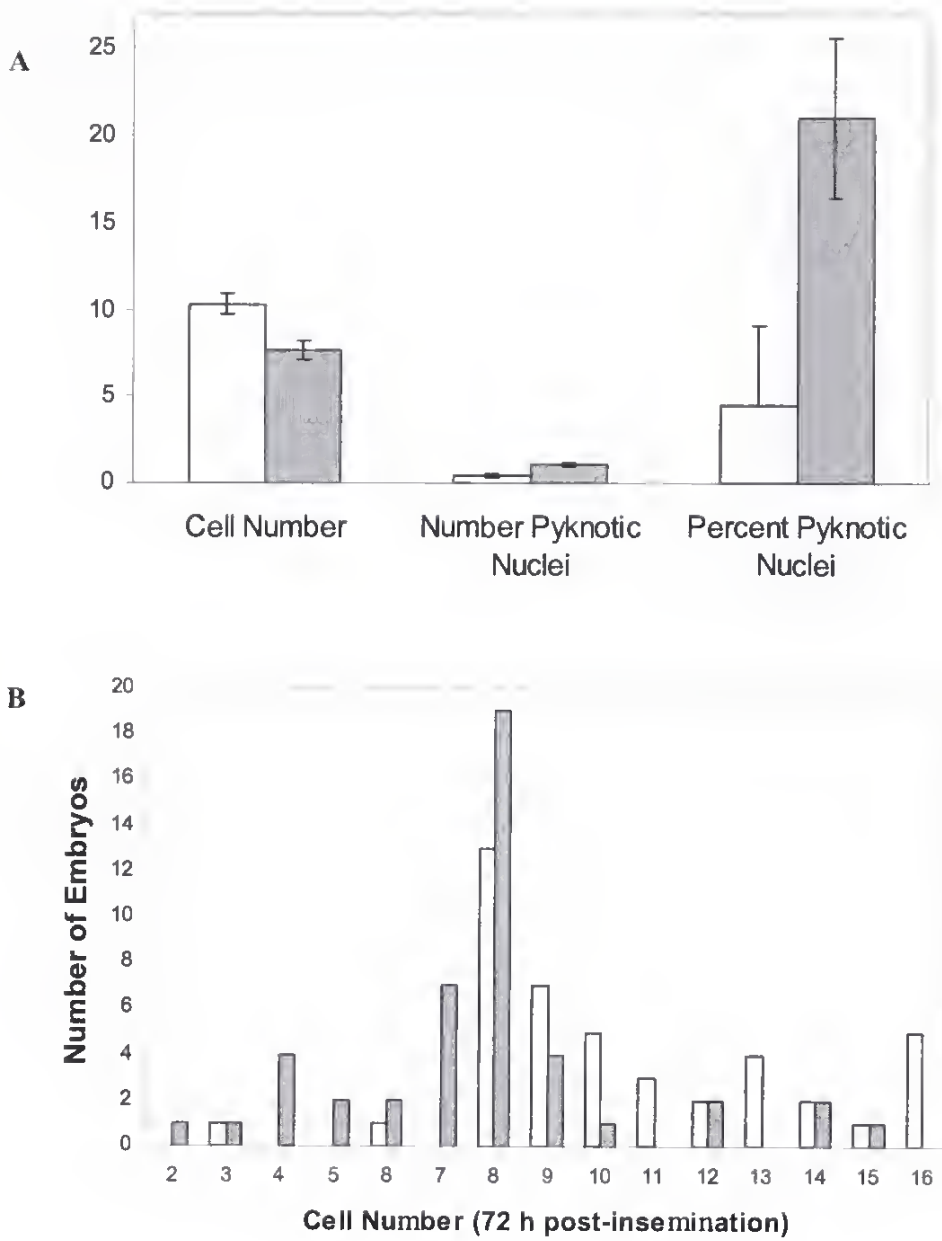


Figure 4-5. Effects of heat shock at the two-cell stage on number of total and pyknotic nuclei 72h after insemination. Data in panel A are least-squares means ± SEM for total number of nuclei and number and percent of pycnotic nuclei as affected by heat shock. Panel B shows the distribution of cell number at 72 hpi. Open bars represent control embryos while grey bars represent heat-shocked embryos. The experiment was replicated four times with 45 embryos per treatment.

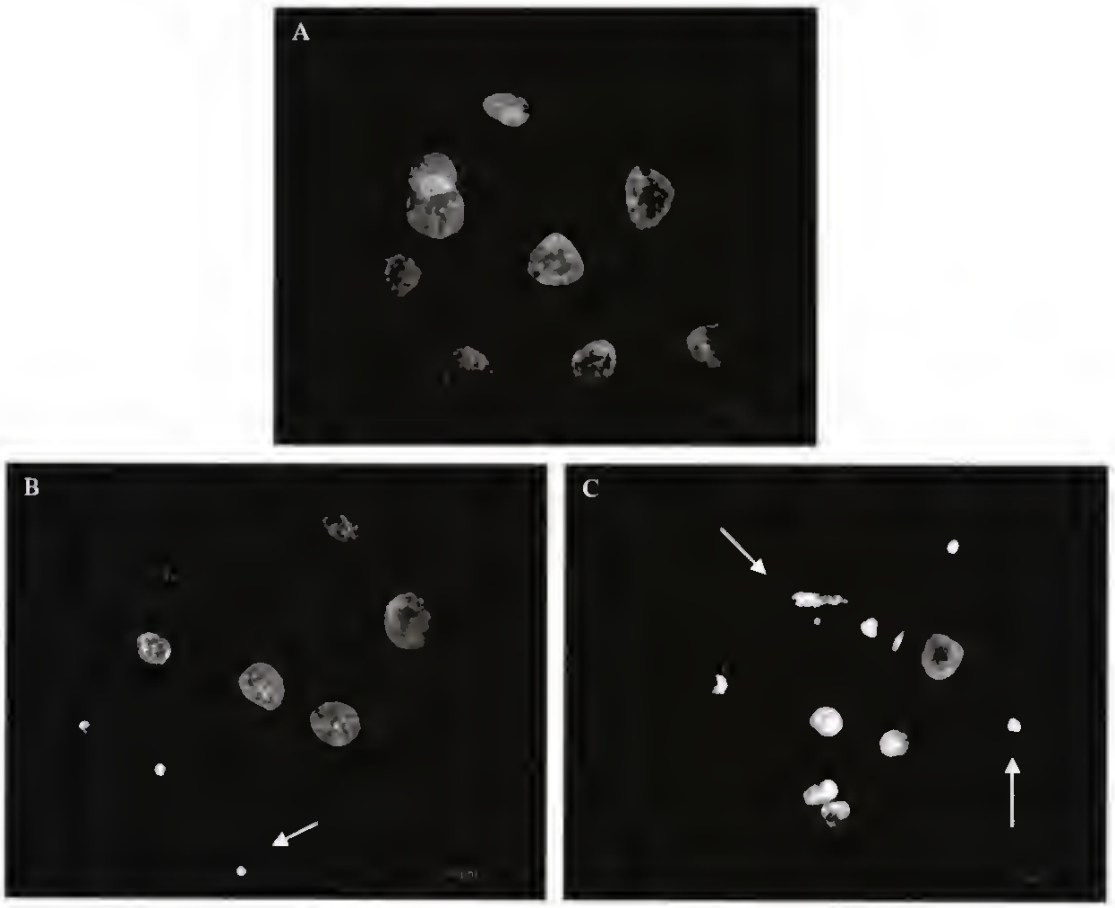


Figure 4-6. Effects of heat shock at the two-cell stage pyknotic nuclei 72h after insemination. The photomicrographs in panel A are of representative images of control (panel A) or heat-shocked (panel B-C) embryos stained with Hoechst nuclear stain at 72 hpi. Arrows point to pyknotic nuclei. The sperm heads on the zona pellucida were digitally removed from the pictures.

Discussion

While it is well established that exposure to a mild heat shock decreases development of preimplantation embryos (Alliston et al., 1965; Elliot et al., 1968; Wattemann and Bazer, 1985; Gwazdauskas et al., 1992; Aréchiga et al., 1995; Aréchiga and Hansen, 1998; Chapter 2) the mechanisms by which development is inhibited are not known. Increased production of free radicals has been implicated as a cause of the developmental block caused by heat shock in mice (Aréchiga et al., 1995, Aréchiga and Hansen, 1998; Ozawa et al., 2002). Nonetheless, results from the present experiments fail to support a role for free radicals in actions of heat shock on development in cattle since low oxygen content did not reduce the effects of heat shock and because heat shock did not lower glutathione content of the embryo. Heat shock can also cause disruption of organellar structure in embryos. In cattle, exposure to 41.0°C for 6 h causes redistribution of organelles away from the periphery (indicative of cytoskeletal rearrangement) and an increase in the number of swollen mitochondria (Chapter 3). Present results indicate that the heat-induced alterations in mitochondrial structure do not lead to immediate disruption of oxidative phosphorylation because heat shock did not cause a significant reduction in oxygen consumption or ATP content at the two-cell stage. Indeed, present findings illustrate that the inhibition of development caused by heat shock at the two-cell stage is not immediate. Rather, embryos develop to the eight-cell stage where they become blocked and develop pyknotic nuclei. Perhaps damage to the mitochondria at the two-cell stage is not sufficient to block development immediately because oxidative phosphorylation is low at this time (Thompson et al., 1996) and one response to damaged mitochondria, apoptosis, cannot be activated by heat shock in two-

cell embryos (Paula-Lopes and Hansen, 2002a). However, damage to the mitochondria or other changes occurring at the two-cell stage make the embryo unable to proceed past the eight-cell stage. This stage may be particularly difficult for the embryo to traverse since it is when the embryo is undergoing major changes in function characterized by the process of embryonic genome activation (Memili et al., 1998).

The conclusion that effects of heat shock on development do not involve oxygen free radicals is a tentative one since no direct measurement of reactive oxygen species was made. If an increase in free radicals was important for the inhibition of development, however, one would expect that effects of heat shock would be greater in the presence of high oxygen. Many oxidases that produce reactive oxygen species are known to be oxygen dependent (Fridovich, 1964; Cohen and Fridovich, 1971) and amounts of oxygen radicals within mouse embryos is dependent upon oxygen environment (Goto et al., 1993). Embryos cultured in low oxygen were not less sensitive to heat shock, however. In fact, the decrease caused by heat shock was slightly greater for embryos cultured in low oxygen than in air because overall rates of development at 38.5°C were greater. In an earlier study, exposure of embryos to a 5% O_2 environment during heat shock did not lessen the detrimental effects of heat shock when compared to control embryos (Chapter 2). In that study, embryos were cultured in low oxygen only during heat shock.

The lack of difference in the content of glutathione between heat-shocked and control embryos is additional evidence that free radicals are not a major source of damage to the two-cell embryo. Consistent with this idea is an experiment in which addition of various antioxidants did not reduce the effects of heat shock on bovine embryonic

development (Ealy et al., 1995; Paula-Lopes et al., 2003a). In contrast to the situation in bovine embryos, there is abundant evidence that free radicals play a role in disruption of development caused by heat shock in the mouse (Noda et al., 1991; Aréchiga et al., 1995; Ozawa et al., 2002). One could speculate that the two-cell mouse embryo is more sensitive to free radical damage than the two-cell bovine embryo because oxidative phosphorylation is more active at this stage in the mouse (Trimarchi et al., 2000).

In an earlier study, 15% of mitochondria of two-cell embryos exposed to 41.0°C for 6 h had become swollen (Chapter 3). Mitochondrial swelling is the result of a change in the conductance of the mitochondrial permeability transition pore to allow unselective diffusion of large molecules into the matrix (Ichas and Mazat, 1998). A major consequence of the mitochondrial permeability transition is the uncoupling of oxidative phosphorylation as a result of a collapse of the membrane potential and pH gradient (Duchen, 1999; Halestrap et al., 2002; Skulachev, 2002) and a drop in the ATP/ADP ratio (Duchen, 1999). Eventually, the swollen mitochondrion undergoes death in a process called mitoptosis (Skulachev, 2002). Given these effects of heat shock on mitochondrial function, it was hypothesized that heat shock would result in a reduction in the oxygen consumption. Since the contribution of ATP from glycolysis is low in the early preimplantation embryo and energy is largely generated by oxidative phosphorylation (Houghton et al., 1996; Thompson, 2000), it was also expected that heat shock would cause the intracellular content of ATP to decline. The rate of oxygen consumption in embryos at 38.5°C was 2.8×10^{-13} moles/s. This value is similar to values previously reported for the bovine embryo (Shiku et al., 2001). Heat shock did not significantly reduce oxygen consumption although it was numerically lower for embryos at 41.0°C.

Moreover, heat shock did not affect ATP content and so it can be concluded that heat shock did not cause major changes in energy metabolism at this stage.

One possibility is that the loss of mitochondria by mitoptosis (Chapter 3) did not cause a reduction in ATP content because either ATP utilization was reduced or heat shock increased activity of enzymes involved in oxidative phosphorylation. Also, a decrease in mitochondrial ATP synthesis could be compensated for by increased production of ATP by glycolysis, as suggested for some other cells types exposed to heat shock (Macouillard-Poulletier de Gannes et al., 2000). The low activity of the glycolytic pathway in the preimplantation embryo (Thompson, 2000) makes this explanation unlikely. What is more likely is that heat shock did not have an immediate effect on oxygen consumption or ATP content because mitochondria of the two-cell bovine embryo are immature and not an important contributor to energy metabolism. Early-cleaving embryos contain structurally undifferentiated mitochondria (Van Blerkom et al., 1990; Plante and King, 1994) which have a low ATP synthetic ability (Rozell et al., 1992; Khurana and Niemann, 2000) and do not replicate (Smith et al., 2000; Cummins, 2001). Consequently, oxygen consumption is low during the early preimplantation stages (Leese, 1991; Thompson et al., 1996).

The observation that the two-cell embryo did not immediately stop developing after heat shock but rather became blocked at the eight-cell stage is consistent with the idea that damage to the mitochondria has no immediate effect on the embryo but that heat-induced changes in mitochondria or other cellular processes becomes catastrophic as metabolic demands of the embryo increase because of increased transcription (Memili and First, 2000). Blastomeres with a deficient number of mitochondria may overproduce

ATP to meet the metabolic need of the blastomere, and this overproduction could itself trigger mitochondrial demise and send the cell into apoptosis (Skulachev, 2002; Waterhouse, 2003). The increase in cells with pyknotic nuclei could be evidence of apoptosis triggered as a result of mitochondrial damage (Burgoyne, 1999). That only some nuclei were pyknotic could, in part, reflect asymmetrical distribution of mitochondria during cleavage. Such a phenomenon has been reported for human cleavage-stage embryos, resulting in some blastomeres having reduced mitochondrial inheritance and diminished ATP capacity (Van Blerkom et al., 2000).

In summary, exposure of bovine embryos at the two-cell stage did not appear to cause a block to development by increasing oxygen free radicals or causing an immediate reduction in oxidative phosphorylation. Embryos did not become inhibited in development immediately after heat shock but rather progressed to the eight-cell stage before blocking in development and developing pycnotic nuclei. It is likely that mitochondrial damage or other alterations in the embryo brought about by heat shock at the two-cell stage makes the embryo unable to proceed past the eight-cell stage, a critical time in embryo development when there is a surge in embryonic transcription (Barnes and Eyestone, 1990; Memili et al., 1998), and heat-induced apoptosis is possible (Paula-Lopes and Hansen, 2002a).

CHAPTER 5
ACTIONS OF HEAT SHOCK ON REORGANIZATION OF MICROFILAMENTS AND MICROTUBULES IN TWO-CELL BOVINE EMBRYOS

Introduction

The developmental program and survival of the preimplantation mammalian embryo is very susceptible to disruption by elevated ambient temperature (Alliston et al., 1965; Edwards and Hansen, 1997; Ozawa et al., 2002; Chapter 2; Chapter 3). Temperatures as low as 39.0°C in the mouse (Gwazdauskas et al. 1992), 40.0°C in sheep (Dutt, 1963), and 41.0°C in cattle (Ealy et al., 1993; Chapter 2) can disrupt development and little is known about how such a mild heat shock can compromise cellular function of the preimplantation embryo. In bovine preimplantation embryos, heat shock can cause decreased protein synthesis (Edwards et al., 1997), induction of apoptosis (after the 8-cell stage, Paula-Lopes and Hansen, 2002a), increased synthesis of heat shock protein 70 (Edwards et al., 1997; Chandolia et al., 1999a), mitochondrial swelling (Chapter 3), and movement of organelles away from the plasma membrane (Chapter 3). The translocation of organelles towards the center of the blastomere is suggestive of cytoskeletal reorganization because organelles are tightly associated with cytoskeletal elements (Stebbings, 1996; Kamal and Goldstein, 2000; Goode et al., 2000).

It is well documented that heat shock (albeit of a higher temperature than that disrupting function of embryos) induces alterations to the cytoskeleton. The specific cytoskeletal element most sensitive to heat shock varies with cell type. For example,

exposure of mouse epithelial cells to elevated temperatures changed the organization of keratin filaments and actin filaments but had no effect on microtubules (Shyy et al., 1989). Similar results were observed in 9L cells where heat shock caused collapse of microfilaments and intermediate filaments but had only slight effects on microtubules (Wang et al., 1998). In contrast, microtubules were disrupted by heat shock in Chinese hamster ovary cells (Coss et al., 1996) and mouse 3T3 cells (Lin et al., 1982).

The objective of the present study was to determine the involvement of disruption in the microtubule and microfilament cytoskeleton in heat-shock induced redistribution of organelles and other ultrastructural changes in the bovine two-cell embryo.

Materials and Methods

Materials

All chemicals, except otherwise specified, were from Sigma (St. Louis, MO). Reagents for in vitro production of embryos were obtained as described elsewhere (Chapter 2; Chapter 3). Percoll was from Amersham Biosciences (Piscataway, NJ). Penicillin-streptomycin and SP-TL, Hepes-TL, and IVF-TL culture media were from Cell and Molecular Technologies (Lavallette, NJ) and were used to prepare SP-TALP, Hepes-TALP, and IVF-TALP (Parrish et al., 1986). Potassium simplex optimized medium (KSOM; formulation MR-020-D) was also from Cell and Molecular Technologies and was used to prepare KSOM-BE1 by the addition of 3 mg/ml essentially fatty acid free Fraction V BSA, 2.5 μg/ml gentamicin, essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) as described elsewhere (Soto et al., 2003). Bovine steer serum was from Pel Freez Biologicals (Rogers, AK).

Glutaraldehyde, 16% (v/v) formaldehyde, osmium tetroxide, ethanol, acetone, EMbed-812, uranyl acetate, Reynolds' lead citrate, Butvar, and copper grids were from Electron Microscopy Sciences (Fort Washington, PA). Alexa Fluor 594 phalloidin, and ProLong antifade solution were from Molecular Probes (Eugene, OR).

Production of Embryos

Procedures for oocyte maturation, fertilization, and embryo culture were as described previously (Chapter 3 et al., 2003). Briefly, oocytes obtained by slashing the ovary were matured for 21 h and then inseminated with a cocktail of Percoll-purified spermatozoa from three different Angus bulls; a different group of bulls was used for each replicate. At 18 to 20 h after insemination, putative zygotes were denuded of cumulus cells by suspension in Hepes-TALP medium containing 1000 units/ml of hyaluronidase type IV and vortexing in a microcentrifuge tube. Presumptive zygotes were then placed in groups of ~30 in 50 μl microdrops of KSOM-BE1. Two-cell embryos were selected at ~ 28 h after insemination and placed in fresh 50 μl microdrops of KSOM-BE1 for experiments. All cultures at 38.5°C were performed in an environment of 5% (v/v) CO_2 in humidified air. The percent CO_2 was adjusted in the incubators used for heat shock treatments (7% and 8% CO_2 for 41.0 and 43.0°C, respectively) to maintain medium pH at ~7.4 (Chapter 2). The temperature of all incubators was calibrated routinely to assure accuracy of treatments.

Effects of Microfilament and Microtubule Depolymerization on Heat Shock-Induced Movement of Organelles

This experiment was designed as a 3x3 factorial design with 3 inhibitor treatments - vehicle (0.1% ethanol), 1 μM latrunculin B (a potent inhibitor of actin polymerization which causes a shortening and thickening of stress fibers; Spector et al., 1989), and 10

nM rhizoxin (inhibits microtubule assembly and also depolymerizes pre-formed microtubules; Takahashi et al., 1987), and three temperatures - 38.5°C (i.e., homoeothermic body temperature of the cow), 41.0°C (characteristic temperature for heat-stressed cows) or 43.0°C (severe heat shock). Approximately 28 h post-insemination, two-cell embryos were collected and placed in fresh 50 µl microdrops of KSOM-BE1 with or without cytoskeletal inhibitors (5-10 embryos per drop). Embryos were cultured for 6 h at one of the three experimental temperatures. Immediately after treatment, embryos were processed for electron microscopy. The experiment was replicated three times on different days. For statistical analysis, electron micrographs of one embryo per replicate per treatment (selected at random) were used to take measurements on cellular morphology.

Effects of Microtubule Stabilization on Heat Shock-Induced Movement of Organelles

This experiment utilized a 2x3 factorial design with inhibitor treatments consisting of vehicle (0.1% dimethyl sulphoxide; DMSO) or 1 µM paclitaxel (promotes the formation of highly-stable microtubules that resist depolymerization; Schiff and Horwitz, 1980) and temperatures of 38.5, 41.0, or 43.0°C. Two-cell embryos (5-10/replicate) were cultured in microdrops of KSOM-BE1 with or without paclitaxel for 6 h at one of the three temperatures listed above. Immediately after treatment, embryos were processed for electron microscopy. The experiment was replicated three times on different days. For statistical analysis, electron micrographs of one randomly selected embryo per replicate per treatment were used to take measurements on cellular morphology.

Electron Microscopy and Morphometric Analysis of Electron Micrographs

Procedures for embryo fixation and processing were conducted as described previously (Chapter 3). For each two-cell embryo, morphometric analysis was performed on four electron micrographs obtained from one randomly chosen blastomere. Morphometric analysis was performed using an SIS Megaview III camera and software (Soft Imaging System, Lakewood, CO). The distance from the plasma membrane to the organelle-containing region of the cytoplasm was measured at 10 random locations from each of four pictures per embryo (i.e., 40 random measurements/embryo). The average of these measurements was used for statistical analysis. Electron micrographs were also visually assessed to determine effects of heat shock on other aspects of cellular morphology.

Localization of Microfilament by Fluorescence Microscopy

Two-cell embryos were cultured at 38.5 or 43.0°C for 6 h. Immediately after heat shock, the zona pellucida of each embryo was removed by incubation in 0.5% (w/v) protease from *Streptomyces griseus* in phosphate-buffered saline (10 mM - PBS; $NaPO_4$, pH 7.4 containing 0.15 M NaCl) supplemented with 1 mg/ml polyvinyl pyrrolidone (PBS+PVP) for approximately 1 min at 39.0°C. Embryos were thoroughly washed in KSOM-BE1 prior to a 10 min fixation with 3.7% (v/v) formaldehyde in 0.2 M $NaPO_4$, pH 7.4 containing 0.15 M NaCl at 39.0°C. After fixation, embryos were washed three times in PBS+PVP. Embryos were then permeabilized with 0.1% Triton-X 100 for 5 min followed by three washes with PBS+PVP. Embryos were incubated in 10% (v/v) normal goat serum in PBS (PBS+S) at room temperature for 30 min followed by incubation in Alexa Fluor 594 phalloidin for 30 min at room temperature. The phalloidin was prepared

by diluting 5 µl methanolic phalloidin stock solution (prepared as per manufacturer's specification) in 200 µL PBS+S. Embryos were then washed three times in PBS+S before a second 10 min fixation in 3.7% (v/v) formaldehyde, washing three times in PBS+PVP and placement on a glass slide with a cover slip mounted with 5 µL ProLong antifade solution. Negative controls were stained similarly except that phalloidin was omitted from the solution. Embryos were viewed using a high-resolution black and white AxioCam MRm digital camera connected to a Zeiss Axioplan2 epifluorescent microscope (Carl Zeiss, Gottingen, Germany). Images were acquired using AxioVision software (Zeiss). The experiment was replicated 3 times with a total of 15-16 embryos per treatment. Attempts were made to localize microtubules by the use of fluorescent microscopy with anti-bovine anti-tubulin mouse monoclonal antibody but acceptable localization could not be obtained.

Statistical Analysis

Treatment effects on the distance from the plasma membrane to the outermost organelles were analyzed by least-squares analysis of variance using the Proc GLM procedures of SAS (SAS for Windows, Version 8e, Cary, NC). The model included effects of temperature, inhibitor treatment, and temperature x treatment. Data are presented as least-squares means ± SEM.

Results

Effects of Microfilament and Microtubule Depolymerization on Heat Shock-Induced Changes in Ultrastructure

Two-cell bovine embryos are mainly composed of immature mitochondria and vesicles of unknown composition (Figure 5-1). For embryos cultured at 38.5°C,

organelles were evenly distributed throughout the blastomere and extended to the plasma membrane, regardless of treatment (Figure 5-1A to 5-1C). Heat shock at 41.0 and 43.0°C resulted in movement of organelles away from the periphery of the blastomere to create an area devoid of organelles (Figure 5-1D and 5-1G). Heat shock at 43.0°C (but not 41.0°C) also caused mitochondria to lose their normal morphology so that they became very dark and small (Figure 5-1G). Other effects of heat shock were also evident. For example, a proportion of the mitochondria were swollen at both heat shock temperatures (for example see Figure 5-2B) and the cytoplasm of embryos cultured at 43.0°C had electron dense material (Figure 5-2D). This material was throughout the cytoplasm (Figures 5-1G to 5-1I and 5-2D) and especially in close proximity to the membranes of organelles, except vesicles. Precipitated chromatin could also be observed for embryos at 43.0°C (Figure 5-2F).

Latrunculin B was effective in causing microfilament disruption as determined by fluorescence microscopy of phalloidin-labeled microfilaments (Figure 5-3). The morphology of microfilaments was changed from long, irregularly-shaped thin fibers (Figure 5-3A) to aggregated material with numerous nodule-like structures (Figure 5-3B to 5-3C). A large ring of actin-containing fibers was also present in some blastomeres from latrunculin B-treated embryos (Figure 5-3B). While embryos cultured in vehicle possessed an intense, narrow network of microfilaments underneath the plasma membrane, especially in regions where blastomeres were in apposition (Figure 5-3A), this network was no longer evident in embryos cultured with latrunculin B (Figure 5-3B to 5-3C).

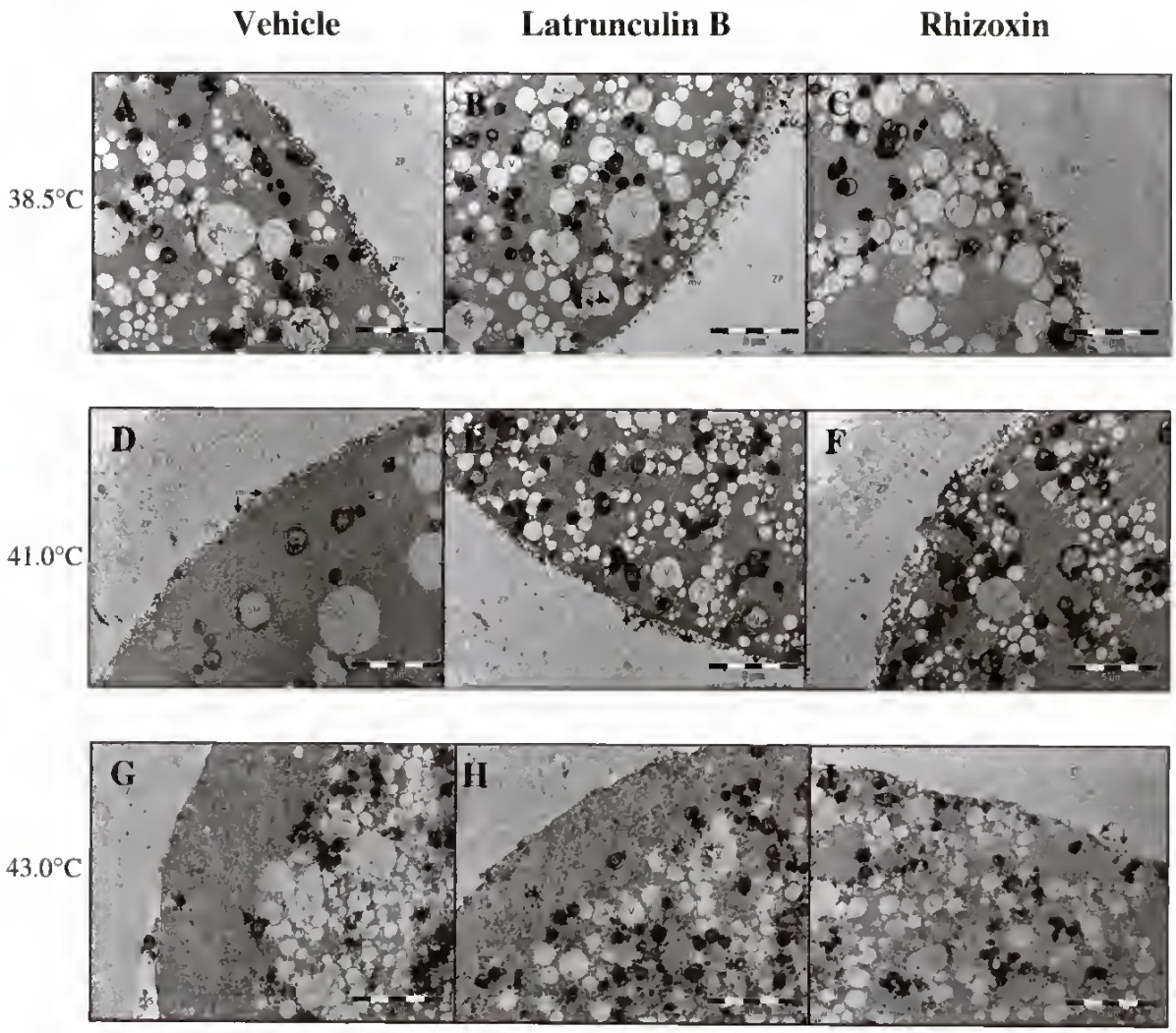


Figure 5-1. Microtubule and microfilament depolymerization agents inhibit heat-shock induced movement of organelles. Shown are representative electron micrographs of two-cell embryos cultured for 6 h at 38.5 (A-C), 41.0 (D-F), or 43.0°C (G-I) with either 0.1% ethanol (vehicle; A, D, G), 1 μM latrunculin B (B, E, H), or 10 nM rhizoxin (C, F, I). Note the large space between the plasma membrane and the nearest organelles in heat-shocked embryos and the reduction in this space by rhizoxin and, at 41°C, . M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli, PVS = perivitelline space.

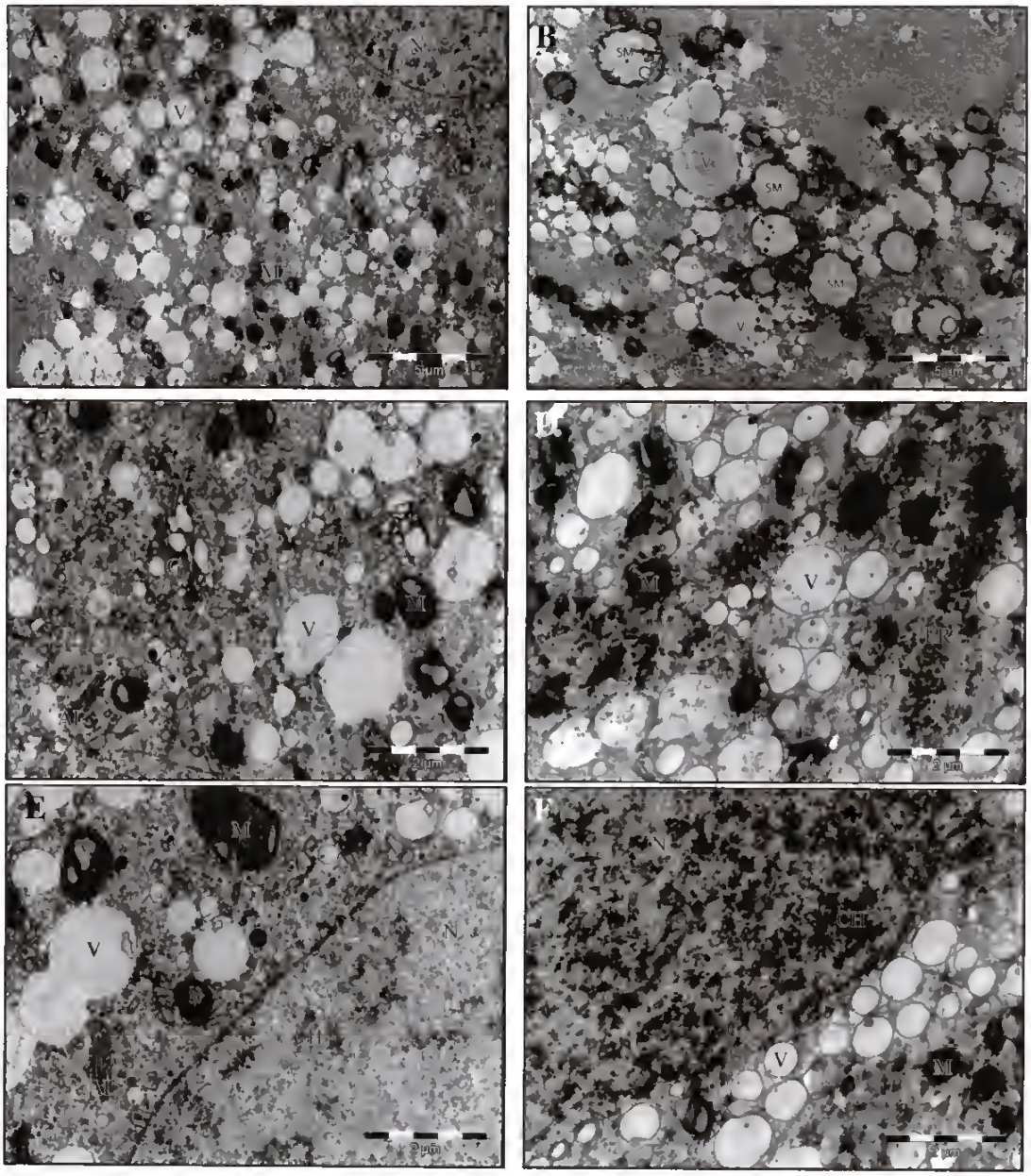
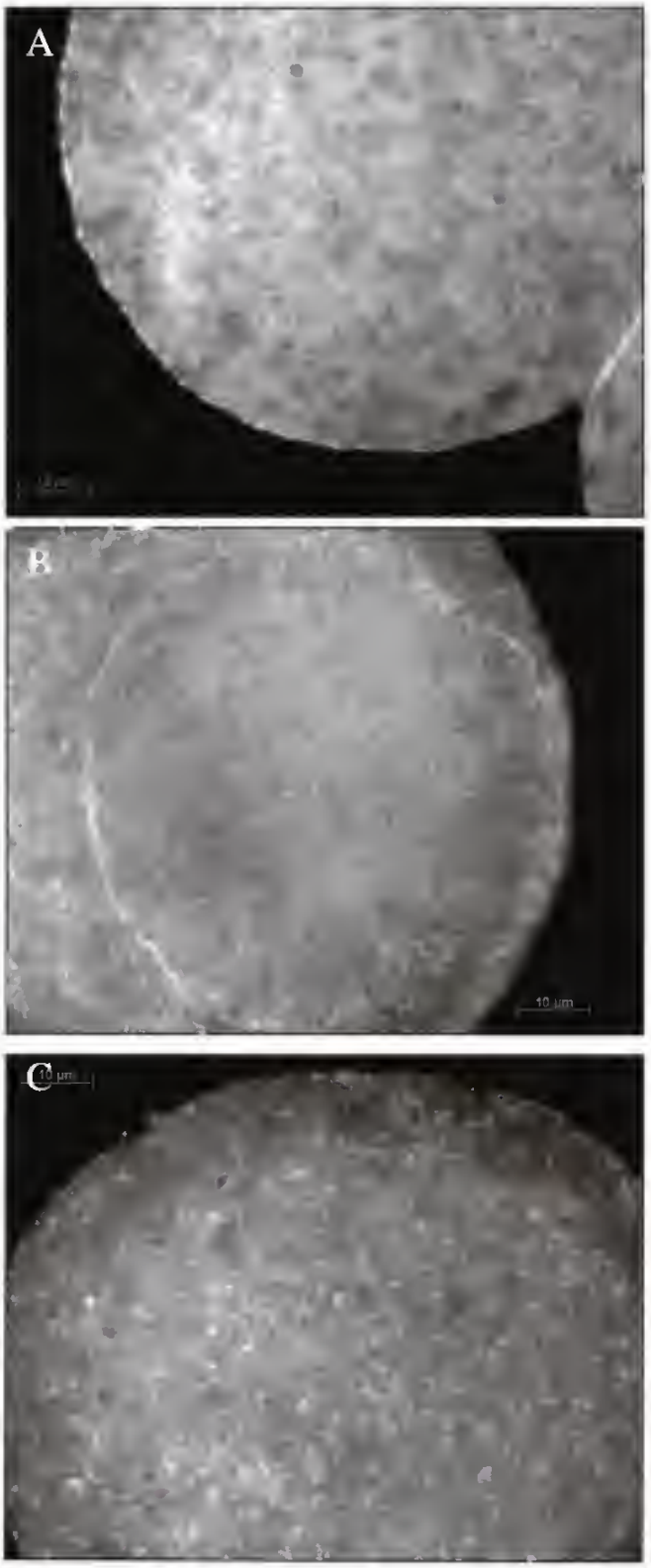


Figure 5-2. Electron micrographs demonstrating alterations in mitochondrial, cytoplasmic, and nuclear morphology as a result of heat shock. Panels A, C, and E are representative micrographs of embryos cultured at 38.5°C. Panel B shows mitochondrial swelling as a result of culture at 41.0°C while panels D and F show protein aggregation and chromatin degeneration of embryos cultured at 43.0°C, respectively. M = mitochondrion, SM = swollen mitochondrion, V = vesicle, CH = chromatin, G = Golgi apparatus, AL = annulate lamellae, N = nucleus.

Figure 5-3. Microfilament disruption by Latrunculin B at 38.5°C. Microfilaments were labeled with Alexa Fluor 594 phalloidin for embryos treated with vehicle (0.1% ethanol; A) and 1 μM latrunculin (B-C).


A
B
10 µm
C
10 µm

The microfilament and microtubule depolymerization agents affected the movement of organelles as a result of heat shock. Results of quantitative analysis of the distance between the plasma membrane and the nearest organelle are illustrated in Figure 4 while representative electron micrographs illustrating the phenomenon are shown in Figure 5-1 (panels D-I). Both depolymerization agents reduced the heat shock-induced movement of organelles (temperature x treatment interaction, $p < 0.001$). However, latrunculin B blocked the effect of heat shock at 41.0°C but not at 43.0°C (see Figures 5-1B, E, H) while rhizoxin blocked the movement of organelles at both temperatures (see Figure 5-1C, F, I).

Rhizoxin, but not latrunculin B, also altered mitochondrial changes induced by heat shock. In particular, the increase in the number of swollen mitochondria caused by heat shock at both 41.0 and 43.0°C was absent for embryos treated with rhizoxin.

Effects of Microtubule Stabilization on Heat-Induced Movement of Organelles

Representative electron micrographs of embryos treated with paclitaxel or vehicle (0.1% DMSO) are shown in Figure 5-5. As for the previous experiment, heat shock caused organelles to redistribute away from the plasma membrane (Figure 5-5A, C, E) and increased the proportion of mitochondria with a swollen morphology. When embryos were cultured at 43.0°C, mitochondria were dark and of small size (Figure 5-5E). Furthermore, several of the embryos at 43.0°C (but not at 38.5 or 41.0°C) possessed structures resembling intra-nuclear intermediate filament bundles (Figure 5-6).

Treatment with paclitaxel caused changes in the ultrastructure of embryos at 38.5°C consistent with its action as a stabilizer of microtubules. In the absence of paclitaxel, the occasional microtubule was observed in the cytoplasm (Figure 5-7A).

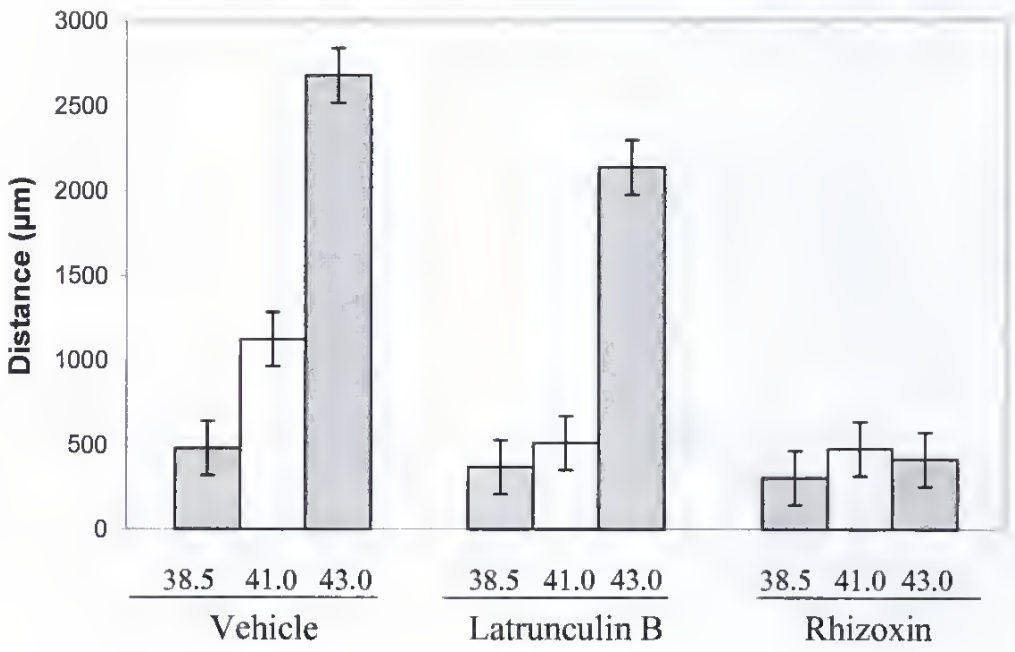


Figure 5-4. Effects of microtubule and microfilament depolymerization on heat-induced movement of organelles. Treatments were vehicle (0.1% ethanol), 1 µM latrunculin B, and 10 nM Rhizoxin. The distance from the plasma membrane to the organelles was taken at 10 random places in 4 pictures per embryo. Bars represent the least-squares means ± SEM of three replicates (one embryo per replicate). There were significant effects of temperature, treatment, and temperature x treatment ($p<0.001$).

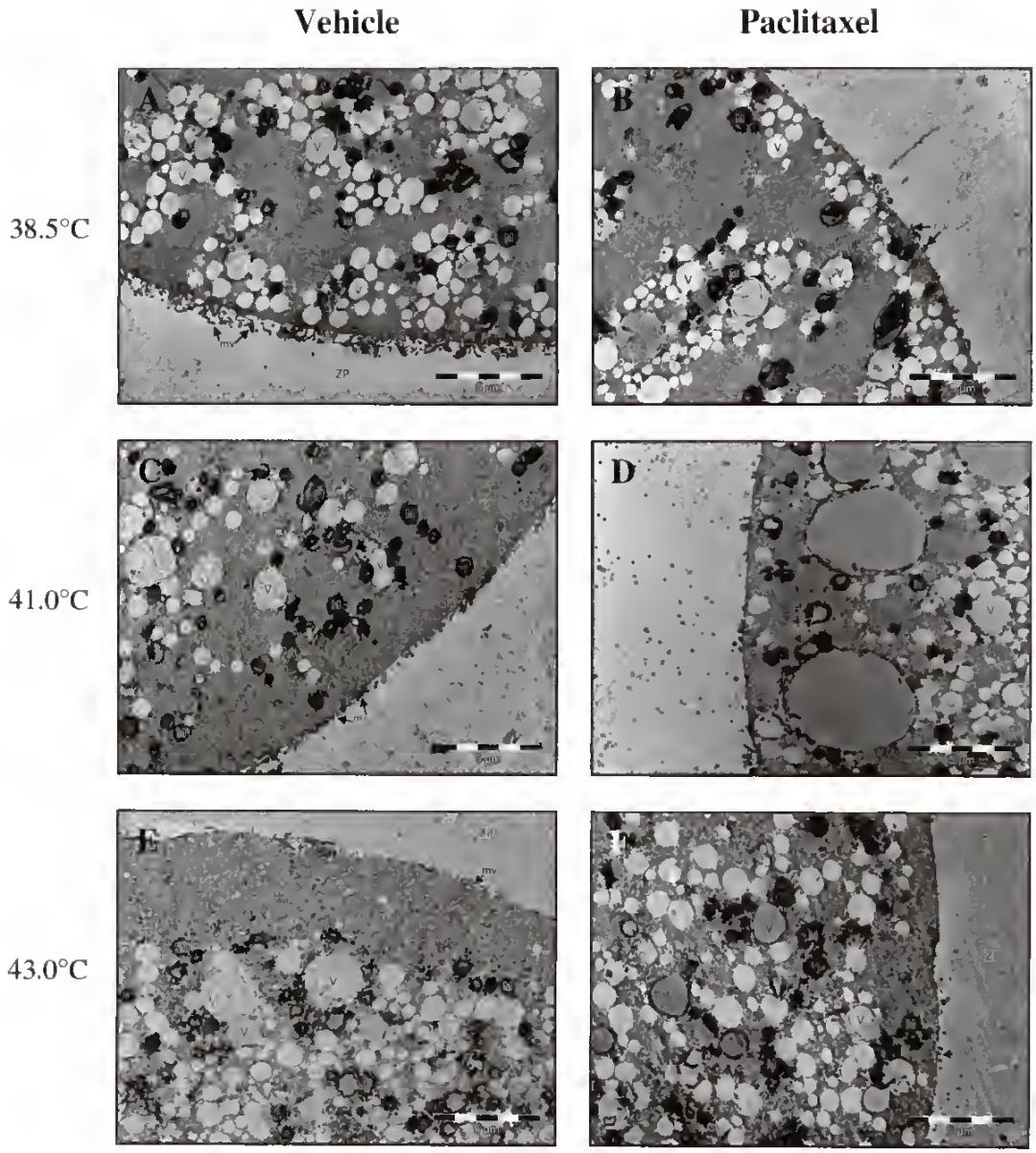


Figure 5-5. Effect of stabilization of microtubules on heat-shock induced movement of organelles. Two-cell embryos were cultured for 6 h at 38.5 (A-B), 41.0 (C-D), or 43.0°C (E-F) with either 0.1% DMSO (vehicle; A, C, E) or 1 μM paclitaxel (B, D, F). M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli, PVS = perivitelline space.

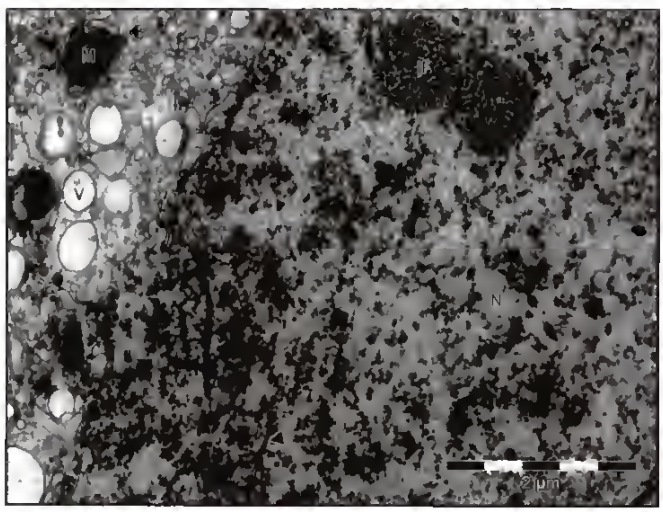


Figure 5-6. Representative electron micrograph illustrating intra-nuclear formation of intermediate filament bundles in embryos cultured at 43.0°C for 6 h. M = mitochondrion, V = vesicle, N = nucleus, IF = intermediate filaments.

In contrast, embryos treated with paclitaxel possessed numerous macrotubules (bundles of microtubules) in the cytoplasm (Figure 5-7C). Tubulin paracrystals were also apparent (Figure 5-7E). In addition, paclitaxel caused alignment of mitochondria into vertical columns organized along the nuclear-plasma membrane axis (Figures 5-5B and 5-8E to F). This observation was unlike other treatments at 38.5°C (vehicle, latrunculin B, or rhizoxin) where mitochondria were evenly distributed throughout the cytoplasm (Figure 5-8A to D). Further, paclitaxel caused accumulation of organelles at the cell margin (Figure 5-5B), and blastomere fragmentation (Figure 5-7D and F).

Results of quantitative analysis to determine whether paclitaxel affected the actions of heat shock to cause organellar movement is shown in Figure 5-9 while representative electron micrographs illustrating the phenomenon are shown in Figure 5-5. The movement of organelles was progressively increased by heat shock in a temperature-dependent manner (temperature; $p < 0.01$). While addition of paclitaxel reduced the distance between the plasma membrane and the nearest organelle ($p < 0.01$), the inhibitor did not prevent movement of organelles away from the plasma membrane in response to heat shock (there was no temperature x treatment interaction). Paclitaxel also did not prevent the induction of mitochondrial swelling caused by heat shock at 41.0 or 43.0°C.

Microfilament Reorganization in Heat-Shocked Cells

Localization of microfilaments using fluorescently-labeled phalloidin was performed to determine whether heat shock of 43.0°C caused changes in microfilament morphology.

Microfilaments at 38.5°C were observed throughout the blastomere as irregularly shaped fibers (Figure 5-10A and C) with a relaxed and entangled (mesh-like) appearance.

Figure 5-7. Representative electron micrographs demonstrating effects of paclitaxel on cellular ultrastructure. Panel A shows the occasional microtubule (arrowhead) found in the cytoplasm of embryos treated with vehicle (Veh.; 0.1% DMSO) and panel B is a representation of the ultrastructure of the cytoplasm of embryos treated with vehicle. Panel B is a micrograph from an embryo treated with vehicle. Panels C-F are images of various structures induced by paclitaxel (Pacl.) including macrotubules (C), tubulin paracrystals (E), and blastomere fragments (D, F). Arrowheads point at microtubules (A), macrotubules (C), or paracrystals (E) and asterisks denote blastomere fragmentation (D, F). M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli, PVS = perivitelline space.

Veh.

Pacl.

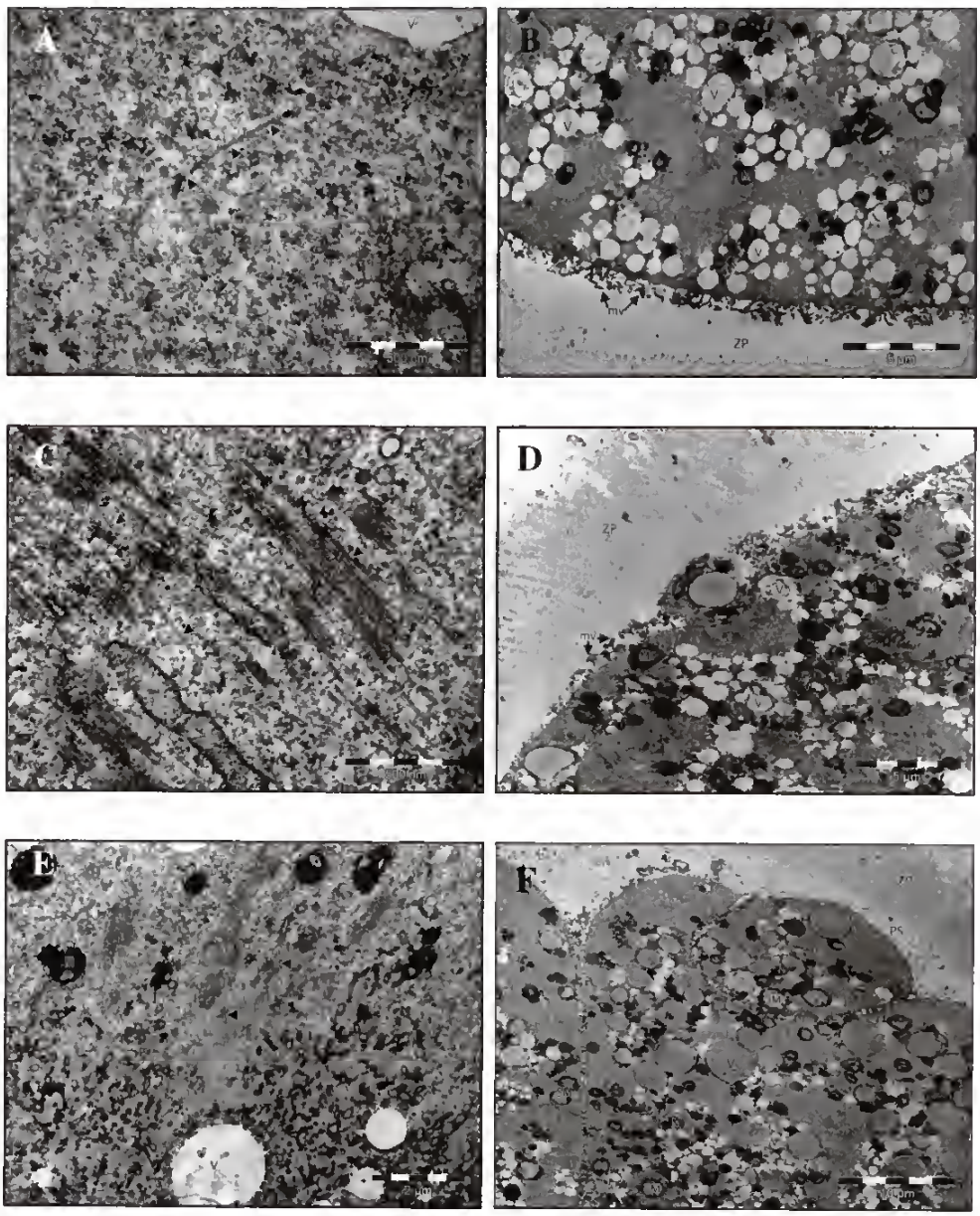
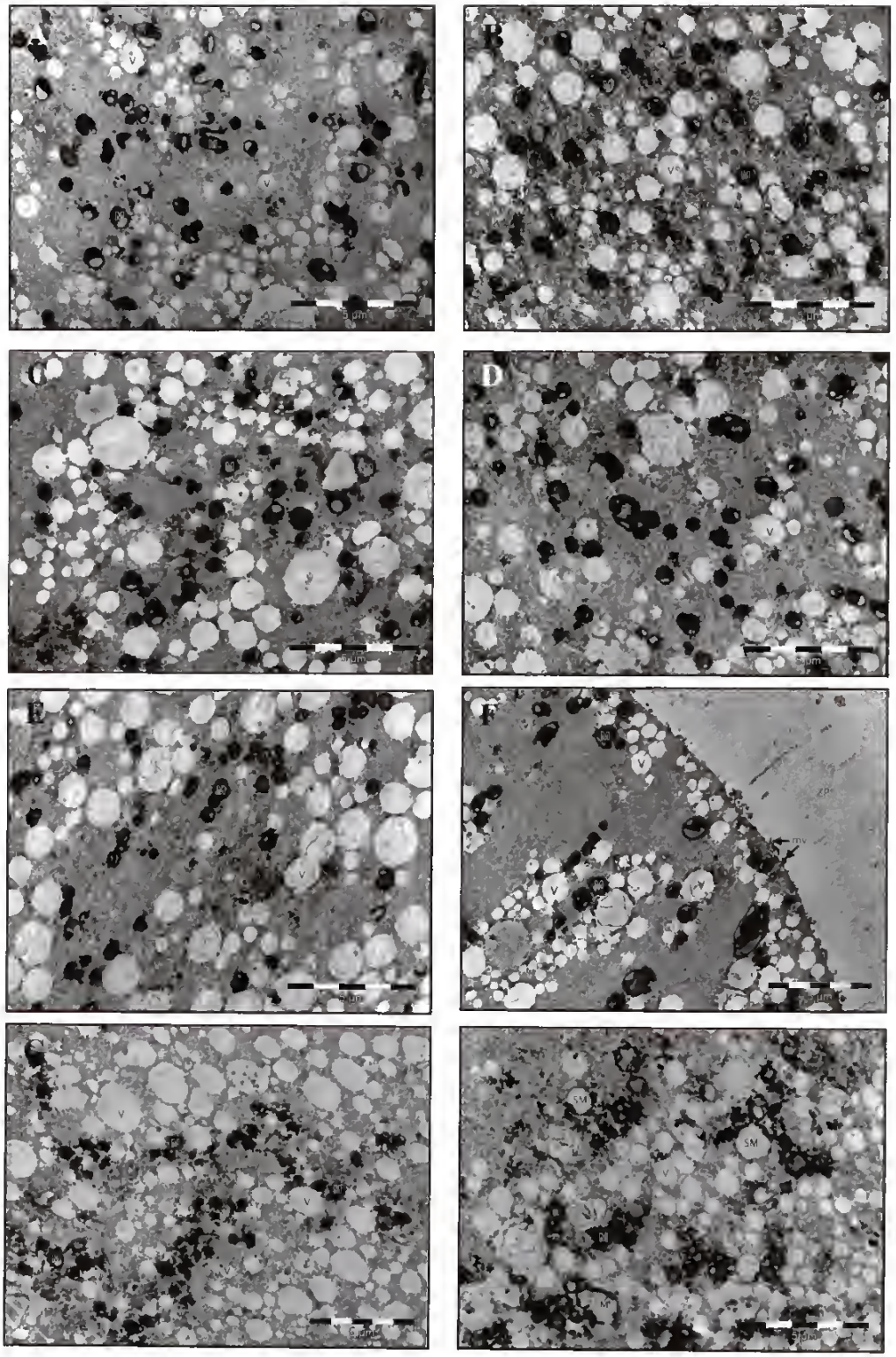


Figure 5-8. Representative electron micrographs illustrating distribution of mitochondria as affected by heat shock and cytoskeletal disruption agents. Shown are images from embryos cultured at 38.5 (A-F) or 43.0°C (G-H) in 0.1% ethanol (A), 0.1% DMSO (B), 1 μM Latrunculin B (C), 10 nM Rhizoxin (D, G), or 1 μM paclitaxel (E-F, H). Note the alignment of mitochondria and vesicles into columns oriented along the nucleus-periphery axis that occur when embryos are cultured in 1 μM paclitaxel (E-F) as compared to those with other treatments (A-D). Culture of embryos at 43.0°C induced mitochondrial swelling in the presence of paclitaxel (H) but not in the presence of the microtubule depolymerization agent rhizoxin (G). M = mitochondrion, SM = swollen mitochondrion, V = vesicle, ZP = zona pellucida, mv = microvilli.


A
B
C
D
E
F
G
H
V
M
SM
mv
CP

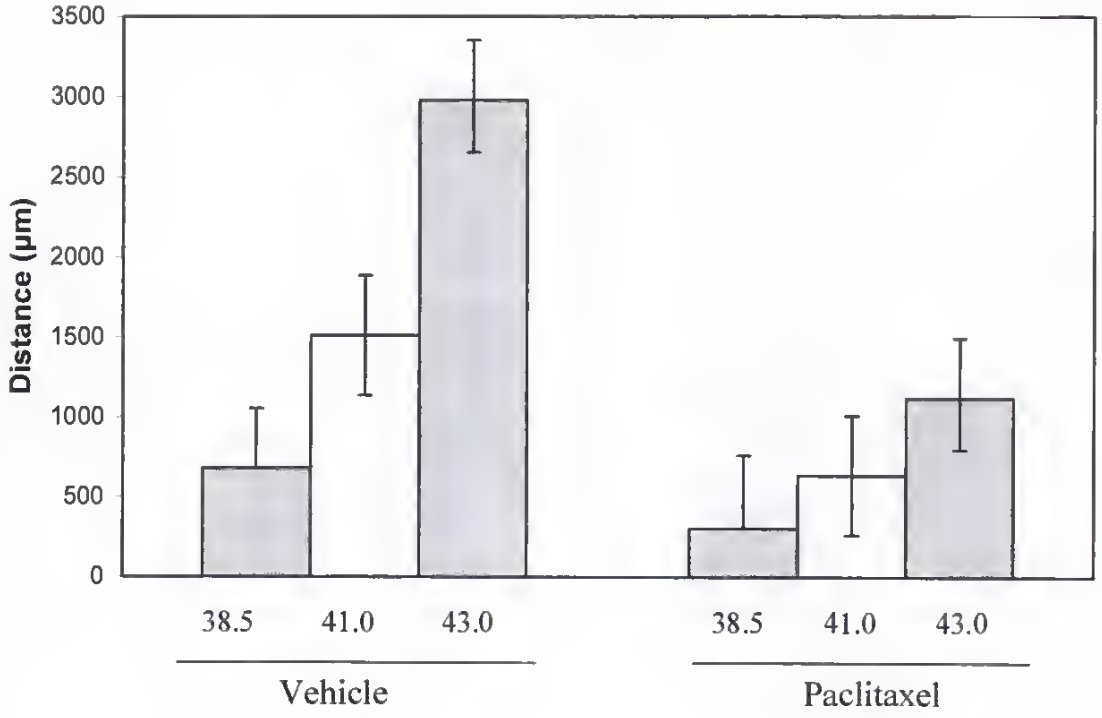


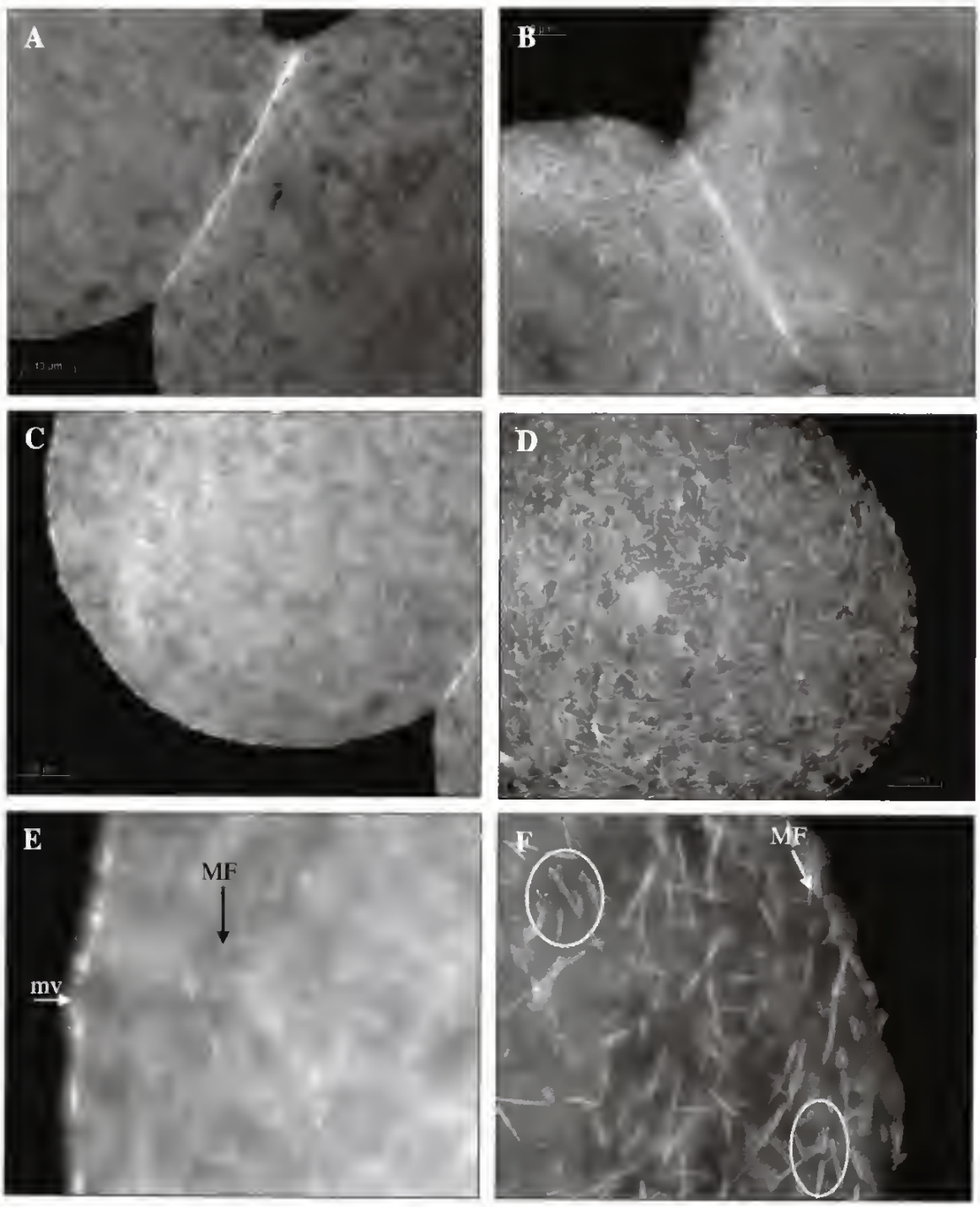
Figure 5-9. Effects of microtubule stabilization on heat induced movement of organelles. Treatments were vehicle (0.1% DMSO) and 1 µM paclitaxel. The distance from the plasma membrane to the organelles was taken at 10 random places in 4 pictures per embryo. Bars represent the least-squares means ± SEM of three replicates (one embryo per replicate). There were effects of temperature ($p < 0.01$) and treatment ($p < 0.01$) but no temperature x treatment interaction.

Microfilaments were particularly prominent in the microvilli around the periphery of blastomeres (Figure 5-10E) and underneath regions of the plasma membrane where blastomeres were apposed (Figure 5-10A). Heat shock of 43.0°C caused reorganization of the actin cytoskeleton (Figure 5-10B and D). In contrast to the more diffuse localization pattern in embryos at 38.5°C, the matrix of microfilaments appeared thinned in heat-shocked embryos and individual microfilaments were more distinct in appearance, with individual short fibers being prominent. While fibers in embryos cultured at 38.5°C were usually straight, with bending seen occasionally, (Figure 5-10E), actin filaments in the heat-shocked embryos were shorter, with a rod-like appearance, and showed breakage (Figure 5-10F). In heat-shocked cells, moreover, the band of microfilaments along the boundary of blastomeres was broader and less dense as compared to control cells and the microvilli were no longer apparent (Figure 5-10A to B)

Discussion

One of the major functions of the cytoskeleton is positioning of organelles in the cell (Kamal and Goldstein, 2000; Goode et al., 2000). The present study demonstrates that the previously-reported movement of organelles away from the periphery of the blastomere induced by heat shock (Chapter 3) is caused by actions of heat shock on microtubule and microfilament organization. In addition, the swelling of mitochondria following heat shock was also found to require intact microtubules. These results indicate that disruption of the cytoskeleton is one of the major effects of heat shock on the preimplantation embryo and is likely to be a key reason for the disruption of

Figure 5-10. Alterations in actin localization patterns caused by heat shock at 43.0°C. Shown are representative epifluorescent images from embryos cultured at 38.5 (A–B) or 43.0°C (C-D) for 6 h. Microfilaments were labeled with Alexa Fluor 594 phalloidin. At 38.5°C, microfilaments were observed throughout the blastomere as irregularly shaped fibers with a relaxed and entangled (mesh-like) appearance (A and C). Microfilaments were particularly prominent in the microvilli around the periphery of blastomeres (E, a digital enlargement of C) and underneath regions of the plasma membrane where blastomeres were apposed (A). In contrast to the more diffuse localization pattern in embryos at 38.5°C, the matrix of microfilaments appeared thinned in heat-shocked embryos and individual microfilaments were more distinct in appearance, with individual short fibers being prominent and sometimes showing breakage (B, D, and F; note F is a digital enlargement of the image in D). Also, the band of fibers along the regions of the cell in apposition to neighbouring cells was thicker and less intense at 43.0°C (B) and a ring of actin delineating the edge of blastomeres not in apposition with other cells could no longer be observed (compare C and D). mv = microvilli, MF = microfilament. Ovals demarcate broken fibers.


A
B
C
D
E
MF
mv
F
MF

development caused by heat shock. Although less extensive than at 43.0°C, cytoskeletal changes in ultrastructure were induced by heat shock at 41.0°C, a temperature frequently experienced by heat-stressed, lactating cows (Ealy et al., 1993; Chapter 3). Thus, the cytopathological changes described here are likely to occur in embryos exposed to elevated temperatures in vivo.

Alterations in cytoskeletal organization in response to heat shock have been reported in a variety of cell lines in culture (Glass et al., 1985; Shyy et al., 1989; Wang et al., 1998). In addition, redistribution of organelles to the perinuclear region has been observed upon heat shock (Shyy et al., 1989). The effect of heat shock on the three components of the cytoskeleton, namely microtubules, microfilaments, and intermediate filaments, is different for different cell types (for a review see Coss and Linnemans, 1996). Moreover, the effects of heat shock on the organization of the cytoskeleton are most likely dependent on the severity of the heat shock and duration of treatment. Here it is demonstrated that the control of organellar movement caused by heat shock depends on temperature. At 41.0°C, organellar movement away from the periphery of the cell could be blocked by either microfilament or microtubule depolymerization. Such a result indicates that damage to both components of the cytoskeleton is required for organellar movement at this temperature. In contrast, at the more severe heat shock of 43.0°C, microtubule depolymerization action with rhizoxin blocked organellar movement but microfilament depolymerization had minimal effects on movement of organelles from the cell periphery. Heat shock at 43.0°C was sufficient to disrupt microfilament structure, as revealed by studies using a phalloidin fluoroprobe to localize actin. The actin rods forming during exposure to 43.0°C have previously been documented in several mouse

cell lines exposed to elevated temperatures (Iida et al., 1986). Presumably, movement of organelles occurred in embryos treated with latrunculin B because the disruption in microtubule structure at 43.0°C was so extensive as to cause movement of organelles even when microfilaments were depolymerized by latrunculin B.

The fact that paclitaxel did not exert the same effects as rhizoxin to block effects of heat shock on redistribution of organelles away from the plasma membrane (or, as discussed below, on mitochondrial swelling) implies that the movement of organelles and swelling of mitochondria induced by heat shock requires intact microtubules but does not require microtubules to be capable of depolymerization. Thus, even though elevated temperature can induce depolymerization of microtubules in cell-free suspension (Turi and Lu, 1981), this phenomenon does not seem to be important for disruption of the microtubule network in the heat-shocked blastomere since changes occurred in the presence of paclitaxel. Perhaps, heat shock causes denaturation of microtubules in a way that alters their shape and position in the cell. Alternatively, heat shock could distort the function of molecular motors on microtubules such as dynein. Such actions of heat shock could conceivably also occur when depolymerization is inhibited by paclitaxel.

Among the effects of heat shock is an increase in the number of swollen mitochondria at both 41.0 and 43.0°C. Mitochondria are arranged along microtubules (Heggeness et al., 1978; Yaffe, 1999) and intracellular distribution and appearance of mitochondria is regulated by association with microtubules (Nangaku et al., 1994; Smirnova et al., 1998) and altered when microtubules are disrupted (Heggeness et al., 1978; Summerhayes et al., 1983; Wang et al., 2003). The importance of microtubules for mitochondrial positioning was illustrated in the current study by the reorganization of

mitochondria into columns along the nuclear-periphery axis upon treatment with paclitaxel. The swelling of mitochondria in response to heat shock appears to require intact microtubules since this phenomenon was blocked by rhizoxin.

Cell-specific actions of heat shock on intermediate filaments probably reflect that intermediate filaments are composed of a multigene family of proteins that are expressed in cell-type specific patterns (Herrmann and Aebi, 2000). Whether heat shock affects intermediate filaments in preimplantation embryos is unknown. Indeed, there is disagreement as to the presence of intermediate filaments in the early mammalian embryo (Gallicano, 2001). Intermediate filaments existing as fibrous sheets of keratin filaments have been described in bovine oocytes and preimplantation embryos (Gallicano et al., 1994) and what appeared to be intra-nuclear intermediate filament bundles were observed in some of the embryos exposed to 43.0°C in the present study. Similar structures composed of cytokeratin have been described in preimplantation embryos of the hamster (Plancha et al., 1991). Perhaps the presence of this fibrillar structure in the nucleus following heat shock is indicative of disruption of intermediate filaments.

Induction of swelling in mitochondria by heat shock is a well-known consequence of heat shock (Cole and Armour, 1988; Welch and Suhan, 1985; Funk et al. 1999) and has been recently reported to occur in bovine two-cell embryos exposed to heat shock (Chapter 3 et al., 2003). The finding that rhizoxin prevented this phenomenon implicates microtubules in this process. Mitochondrial swelling results from loss of membrane potential as a result of the opening of the high conductance permeability transition pores (PTP) on the inner mitochondrial membrane (Halestrap et al., 2002). Opening of the PTP is typically promoted by the accumulation of excessive quantities of Ca^{2+} (Ichas and

Mazat, 1998; Duchen, 1999). The loss of membrane potential allows unselective diffusion of large molecules into the matrix (Ichas and Mazat, 1998) to cause osmotic imbalance and swelling.

There are several possible ways in which microtubules might interact with mitochondria to control opening of the PTP following heat shock. As previously mentioned, opening of the PTP is typically promoted by the accumulation of excessive quantities of Ca^{2+} (Duchen, 1999). Heat shock of 43.0°C results in increases in intracellular calcium ions due to an influx into the cytoplasm from both internal stores and the extracellular medium (Itagaki et al., 1998). The ability of mitochondria to buffer calcium influx in cells is critically dependent upon the microtubule-dependent spatial distribution of mitochondria (Wang et al., 2003) and changes in distribution of mitochondria caused by heat shock may increase vulnerability of this organelle to insults. Alternatively, opening of membrane pores in the mitochondria may depend upon proteins linked to microtubules. One such protein is Bim (Bcl-2-interacting mediator of cell death). This Bcl-2 family protein involved in apoptosis exists either as sequestered to microtubules through binding to the dynein motor complex or in the mitochondrial membrane in association with Bcl-2 (Mollinedo and Gajate, 2003). Upon an apoptotic stimuli like microtubule reorganization, Bim is released from the microtubules and translocates to the mitochondria were it binds Bcl-2, a protein tightly associated with the voltage-dependent anion channel that represents one of the most abundant PTP components (Tsujiomoto and Shimizu, 2002; Carré et al., 2002). Perhaps treatment with rhizoxin interferes with the ability of microtubule reorganization to induce PTP opening through Bim or another protein.

In conclusion, heat shock of two-cell bovine embryos results in changes to the microfilaments, microtubules, and possibly intermediate filaments. In addition, the damage of cytoskeletal components leads to organizational changes in the spatial organization of organelles within the cell and contributes to the swelling of mitochondria. The fact that organelles move towards a perinuclear direction implies that the secretory and absorptive functions of the embryo are impaired by heat shock. Changes in mitochondrial distribution may increase the vulnerability of this organelle to insults such as Ca^{2+}(Ichas and Mazat., 1998; Wang et al., 2003) and facilitate PTP opening and swelling (Ichas and Mazat, 1998; Duchen 1999). Other large-scale pathological changes are seen in blastomeres exposed to 43.0°C including protein and chromatin denaturation as evidenced by electron dense material in the cytoplasm and nucleus.

A major question that arises from these conclusions is the extent to which changes in the cytoskeleton and mitochondrial function caused by heat shock at the physiologically-relevant temperature of 41.0°C contributes to the inhibition of development caused by this mild heat shock. Results from Chapter 4 demonstrate that embryos are capable of continued cleavage after a heat shock of 41.0°C for 6 h before they block in development at the 8-cell stage. Since embryos can recover from heat shock to the point where two more cell divisions are possible, it is likely that much of the damage to the cytoskeleton caused by exposure to 41.0°C can be repaired, as described for other cells (Glass et al., 1985; Macouillard-Poulletier de Gannes et al., 2000). However, repair of the cytoskeleton and perhaps other components of the embryo is not sufficient to allow continued uninterrupted development of the embryo past the eight-cell stage. This carry-over effect of heat shock from the two-cell stage that is expressed at the

eight-cell stage implies that some components of the cellular machinery of the embryo are not completely repaired and eventually prevent the embryo from continuing its developmental program. We suggest that mitochondrial damage at the two-cell stage as a result of microtubule reorganization could result in mitochondrial insufficiency at later stages of development and lead to the arrest of embryonic development and embryo demise.

CHAPTER 6
GENERAL DISCUSSION

Embryonic development is inhibited by exposure of cultured embryos to elevated temperatures. However, previous experiments conducted to demonstrate effects of heat shock on development have used culture temperatures that are higher than body temperatures typically experienced by heat-stressed cows. The goal of this dissertation was to evaluate whether heat shock in the physiologic range (i.e. 41.0°C) disrupted embryonic development and to determine the cellular basis for this effect. The two-cell bovine embryo was chosen because fertility is severely compromised by heat stress in this species (Ealy et al., 1993) and it is at the two-cell stage when the embryo is particularly susceptible to disruption by heat shock (Edwards et al., 1997). Therefore, results are likely to be relevant to the large decline in pregnancy rate seen in heat stressed cows. For many of these studies, a more severe heat shock of 43.0°C was also included to allow comparison of embryonic responses to heat shock with the large literature on cellular effects of heat shock in which temperatures in this range were evaluated. Another goal of this dissertation was to determine if ultrastructural responses to heat shock would be similar between embryos produced in vitro and embryos produced in vivo. This is an important question because it relates to the relevance of the in vitro produced embryo as a model for studying actions of heat shock on embryos. That there might be differences in responses to heat shock between embryos produced in vitro and in vivo is suggested by

the large number of biochemical and cellular differences in function between the two types of embryos (Enright et al., 2000).

The work presented here provides clear support for the idea that some of the infertility caused by exposure of cows to heat stress is a result of direct effects of maternal hyperthermia on embryonic development. This is so because of findings in Chapters 2, 3, and 4 demonstrate that development of two-cell embryos can be disrupted by heat shock in the physiologic range. Moreover, the deleterious consequences of heat shock for the embryo are not the result of artifacts of culture conditions caused by alterations in medium pH or artificially-high oxygen concentration. Indeed, elevated temperature blocked development when the carbon dioxide content of the culture atmosphere was regulated to ensure a constant pH at different culture conditions (Chapter 1). In addition, effects of heat shock on development were similar regardless of whether embryos were cultured in a high or low oxygen environment (Chapters 2 and 4).

The fact that inhibition of embryonic development caused by heat shock is one of the mechanisms for the reduction in fertility in heat-stressed cows has potentially-important practical implications. In particular, it may be possible to develop methods for improving fertility based on reversing some of the cellular damage to the embryo caused by heat shock. Attempts to do so have already been made although these have been unsuccessful. Efforts to reverse effects of heat stress in lactating cows by antioxidant administration have yielded largely negative results (Ealy et al., 1994; Aréchiga and Hansen, 1998; Paula-Lopes et al., 2003a). In addition, efforts to increase resistance of two-cell embryos to heat shock by manipulating antioxidant status (Paula-Lopes et al., 2003a) or heat shock protein synthesis have failed (Al-Katanani and Hansen, 2002)

despite the two-cell embryo's capacity for Hsp70 synthesis (Edwards et al., 1996, Chandolia et al., 1999a). These unsuccessful attempts to improve fertility in heat-stressed cows and heat-shocked embryos may indicate that our understanding of the mechanism by which heat shock compromises embryonic development are not complete. Thus, enhancing knowledge of these mechanisms was an important goal of this thesis.

There is a large literature detailing effects of heat shock on cells. There are at least two reasons to question whether this literature is relevant to the situation for the bovine two-cell embryo, however, as mentioned several times, most studies conducted to determine the pathologies resulting from heat shock have used temperatures ≥ 43.0°C (Ohtsuka et al., 1993; Coss et al., 1996; Wang et al., 1998). These temperatures would be lethal to most mammals and can be so devastating to the normal cellular physiology as to cause necrosis-like changes in the cell. However, the situation is not so simple since, even though the severity of hyperthermia is low in heat-stressed cows (40-41°C; Dunlap and Vincent, 1971; Turner, 1982; Putney et al., 1988; Ealy et al., 1993), the duration of hyperthermia shock is longer (10 h or longer; Chapter 2) than in most studies of cultured cells in which typically the duration of heat shock applied to cells is short (15-30 min). Usually a short severe elevation in temperature is applied to cells. Hence, studies conducted in other organisms and/or cell types, although useful for characterizations of heat-induced pathologies, can only be used to an extent to interpret possible effects of heat shock on early bovine embryo. Secondly, other cells may not be a good model for the bovine two-cell embryo since its cells function differently from most other cells used for heat shock research. At the two-cell stage, the bovine embryo possesses cells that are metabolically (Thompson et al., 1996; Khurana and Niemann, 2000) and transcriptionally

inactive (Thompson, 2000) with immature mitochondria (Plante and King, 1994), and with a shortened cell cycle composed of an S and M phase only (Barnes and Eyestone, 1990). Taken together, the literature on cellular consequences of heat shock can provide a guide to research but there is a compelling need to characterize cellular changes caused by physiologically-relevant heat shocks in the two-cell embryo.

Experiments in Chapters 3 and 5 provided a picture of the alterations in cellular structure and function that are likely to be a major contributor to the block in development caused by heat shock. Specifically, it was found that a physiologically-relevant heat shock of 41.0°C for 6 h induces redistribution of organelles and causes about 15% of mitochondria to undergo swelling reminiscent of mitoptosis (Chapter 3). Evidence was accumulated to demonstrate that organelle redistribution is a result of direct effects of heat shock on microfilament and microtubule reorganization (Chapter 5) and that mitochondrial swelling involves actions of microtubules (Chapter 5). While these ultrastructural changes do not result in a decreased content of ATP nor a reduction in oxygen consumption immediately after heat shock (Chapter 4), embryos are capable of continued development for only two more cleavage divisions before halting at the eight-cell stage. Moreover, at this stage, 22% of the blastomeres of heat-shocked embryos developing to this stage are pyknotic (Chapter 4), suggesting death by apoptosis beginning at the eight-cell stage.

Heat shock has also been demonstrated to cause mitochondrial swelling in other cell types (Cole and Armour, 1988; Welch and Suhan, 1985; Funk et al. 1999). Mitochondrial swelling is the result of calcium overload which switches the mitochondrial conformation of the PTP from a low-conductance state (that allows the

diffusion of small ions like calcium) to a high conductance state (that allows unselective diffusion of big molecules) and thereby causes mitochondrial conformational changes (Ichas and Mazat, 1998). Switching from the low- to the high-conductance state is an irreversible process which leads to release of apoptotic factor such as cytochrome *c* and AIF form the mitochondrial intermembrane space (Ichas and Mazat, 1998). Given these dramatic changes in mitochondrial function caused by swelling, it might be expected that heat shock would decrease ATP content and oxygen consumption. Such responses have been described for other heat-shocked cells (Findly et al., 1983; Calderwood, 1987; Macouillard-Poulletier de Gannes et al., 1998, 2000). It is likely, however, that the lack of effect of heat shock on ATP content and oxygen consumption in two-cell embryos is a reflection of the fact that mitochondria are inactive at this stage of development and not a major source of ATP nor a major consumer of oxygen (Rozell et al., 1992; Khurana and Niemann, 2000).

Nonetheless, the finding that heat shock causes a block to development at the eight-cell stage implies that mitochondrial damage caused by heat shock or other heat-induced alterations in cellular physiology render the embryo unable to proceed past the eight-cell stage. The finding in this thesis that heat-shocked embryos could not continue development past the time when embryonic genome activation occurs (namely the eight-cell stage) has also been observed in the mouse (Elliot and Ulberg, 1971). When mouse embryos were exposed to elevated temperatures in utero during the one-cell stage, there was an increase in the number of two-cell embryos collected and none of these continued development after culture. This is an interesting finding because embryonic genome activation occurs at the late one-cell or two-cell stage in the mouse (Schultz, 1993).

Alterations in cytoskeletal organization in response to heat shock have been reported in a variety of cell lines in culture (Glass et al., 1985; Shyy et al., 1989; Wang et al., 1998). In addition, redistribution of organelles to the perinuclear region has been observed upon heat shock (Shyy et al., 1989). Thus, the two-cell embryo responds similarly to heat shock as cultured cells with respect to these aspects of cellular function (Chapters 3 and 5) despite the difference in temperatures applied. One of the major functions of the cytoskeleton is positioning of organelles in the cell (Kamal and Golstein, 2000; Goode et al., 2000). It is well documented that mitochondria are arranged along the microtubules (Heggeness et al., 1978; Yaffe, 1999) and that intracellular distribution of mitochondria is regulated by the association with microtubules and markedly altered when microtubules are disrupted. Microtubule assembly-disassembly is a temperature sensitive process (Turi and Lu, 1981). Microtubules isolated from calf brain (body temperature of ~38.5°C) will disassemble at 41.0°C and will denature at 43.0°C.

In chapter 5, a direct effect of physiologic heat shock (41.0°C for 6 h) on rearrangement of microtubules was shown when organelle movement was inhibited by the disintegration of microtubules with rhizoxin but not when microtubules were stabilized with the agent paclitaxel. In that study, the actin cytoskeleton was also affected as the movement of organelles was also reduced by microfilament depolymerization with latrunculin B. Rearrangement of cytoskeleton and organelles, especially vesicles, could have a detrimental impact on the embryo by affecting its adsorptive and secretory capacities as well as other functions.

Heat shock could induce mitochondrial swelling by one or more pathways. One possibility is that the observed increase in calcium ions (Itagaki et al., 1998) as a result of

heat shock induces saturation of the internal calcium binding sites of the PTP (Ichas and Mazat, 1998) and induces a change to the high conductance state and swelling. Another possibility, which is supported by the present findings, is that microtubules play an important role. That this is so in embryos was indicated by the observation that mitochondrial swelling did not occur upon heat shock when the microtubule cytoskeleton was depolymerized with rhizoxin (Chapter 5). This phenomenon was particularly evident when embryos were shocked at 43.0°C, a temperature that causes extensive swelling of mitochondria (about 75%; Chapter 3). Tubulin is an inherent component of mitochondrial membranes. Mitochondrial tubulin represents ~2.2% of total cellular tubulin and 0.5% of mitochondrial protein (Carré et al., 2002). In addition, mitochondrial tubulin specifically interacts with the voltage-dependent anion channel (VDAC; Carré et al., 2002), one of the most abundant PTP components (Tsujiomoto and Shimizu, 2002). If tubulin is so prone to denaturation by heat shock as has been documented (Turi and Lu, 1981), perhaps this protein changes conformation as a result of heat shock and induces opening of the PTP. Microtubules could also possibly affect the conformation of the PTP through protein interactions between the mitochondria and the microtubule. Bim (Bcl-2-interacting mediator of cell death) is a Bcl-2 family protein that links microtubules and the onset of apoptosis (Mollinedo and Gajate, 2003). There is evidence that Bim -/- lymphocytes are resistant to apoptotic stimuli induced by microtubule perturbation (Mollinedo and Gajate, 2003). Most of the Bim isoform molecules are bound to the 8kDa subunit of dynein light chain through their dynein binding motif and, so sequestered, are unable to promote apoptosis. Upon apoptotic stimuli, Bim is released from the microtubules and translocates to the mitochondria were it binds Bcl-2, a protein

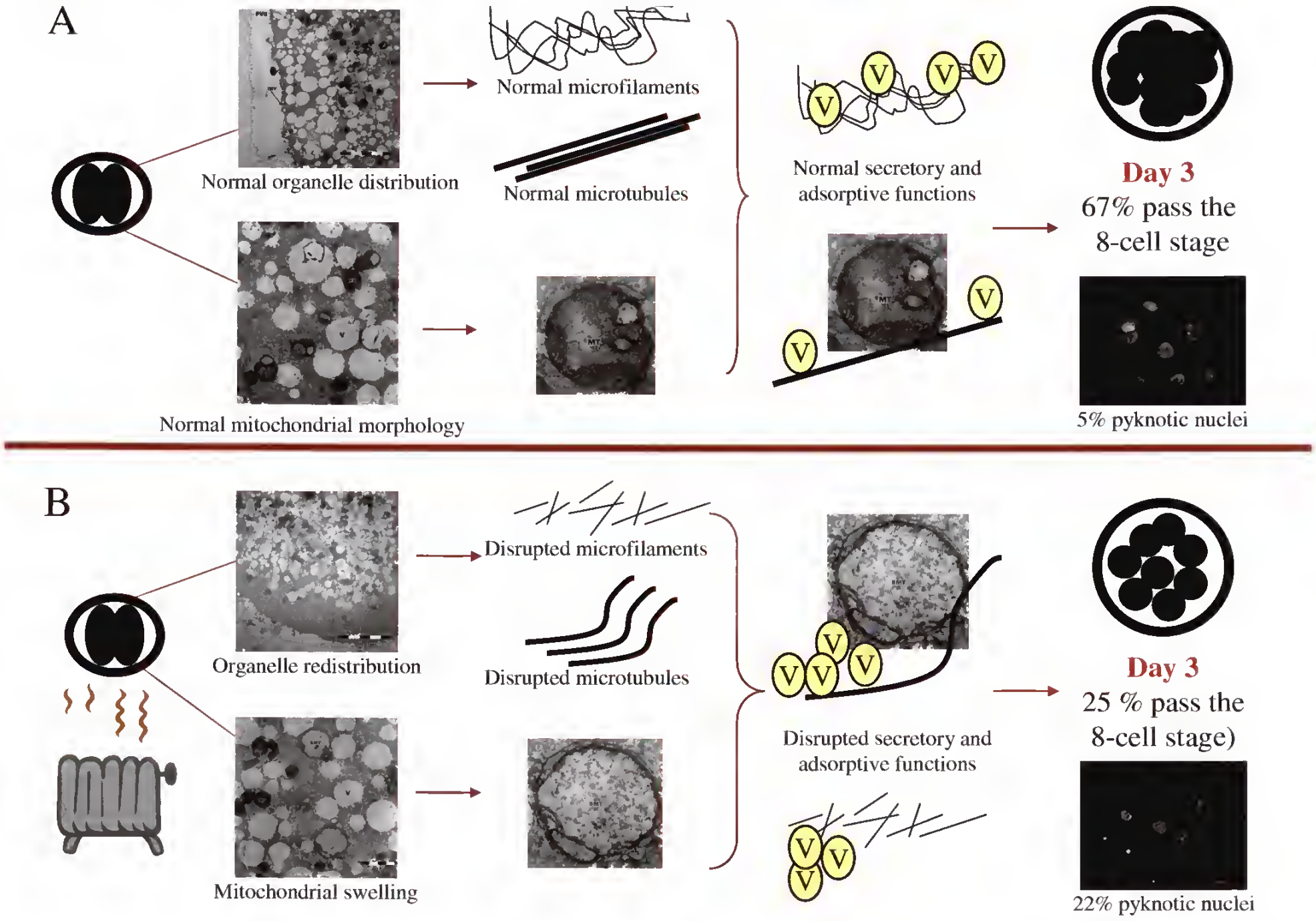
tightly associated with the VDAC (Carré et al., 2002; Tsujiomoto and Shimizu, 2002) thus promoting opening of the PTP, swelling of the mitochondrial matrix, and release of intermembrane proapoptotic protein such as cytochrome *c* that lead to activation of the apoptotic cascade.

Heat-shock induced apoptosis does not occur in the two-cell bovine embryo (Paula-Lopes and Hansen, 2002a). This could mean that insults to the cytoskeleton at the two-cell stage could be transduced to the mitochondria to activate the apoptotic cascade, but since apoptotic pathway is not yet activated at this stage, all that happens is that the mitochondria undergo swelling and die (Skulachev, 2002). As a result of the low metabolic activity of two-cell embryos, death of mitochondria might not pose an immediate problem, as demonstrated in Chapter 4 with the lack of effect of heat shock on ATP content and oxygen utilization. However, this loss of mitochondria could lead to a halt in development and embryonic demise when metabolism increases at the eight-cell stage concomitant with embryonic genome activation (Memili and First, 2000). In addition, induction of apoptosis is possible at this time (Paula-Lopes and Hansen, 2002a) so that potentially apoptosis and pyknosis could be triggered at this stage.

These delayed actions of heat shock on mitochondrial function, as well as the role of cytoskeletal disruption of secretory and absorptive functions of the embryo, could well be among the central causes for the halt in development at the eight-cell stage of embryos that were heat-shocked at the two-cell stage. A model illustrating this idea is depicted in Figure 6-1.

Results in mice (Aréchiga et al., 1995, 1998; Ozawa et al., 2002) have implicated an increased production of free radicals as a cause of the developmental block caused by

Figure 6-1. Proposed model illustrating possible mechanisms involved in demise of the bovine two-cell embryo in response to heat shock in the physiological range. Under normal body temperatures (38.5°C; Panel A), the organelles of two-cell embryos (mostly mitochondria and vesicles of unknown composition) completely occupy the cytoplasm of the blastomeres. This distribution is controlled by movement along cytoskeletal elements, especially microtubules and actin microfilaments. Such an architecture permits secretory and absorptive functions of the vesicles. While mitochondria are immature at this stage, have few cristae, are irregularly shaped, produce low amounts of ATP and consume small amounts of oxygen, they possess a polarized inner membrane and are poised to begin oxidative phosphorylation as the embryo reaches later stages of development. In the absence of heat shock, embryos are capable of sustained development, with about 67% going past the eight-cell stage at Day 3 post-insemination under the culture conditions used in this study. Upon heat shock in the physiologic range (41.0°C for 6 h; Panel B), organelles pull away from the periphery and move in a perinuclear direction as a result of microfilament and microtubule disruption to leave an area devoid of organelles near the edge of the plasma membrane. The movement of organelles disrupts secretory and adsorptive functions. In addition, microtubule reorganization causes about 15% of the mitochondria to switch from a low conductance to a high conductance state so that there is unselective movement of solutes entering the matrix and osmotic imbalance causes mitochondrial swelling and death of the organelle. There appears to be no immediate consequence of mitochondrial death at this stage as measured by ATP content and oxygen consumption. After heat shock, the cytoskeleton is able to reorganize and allow the embryos to continue development for two more cleavage divisions. Nonetheless, the embryo is fatally damaged and most halt development at the eight-cell stage (only 25% develop past this stage under culture conditions used here). At this stage, damage caused earlier leads to nuclear pyknosis probably because of apoptosis caused by mitochondrial damage at the two-cell stage.


A
Normal organelle distribution
Normal microfilaments
Normal microtubules
Normal mitochondrial morphology
Normal secretory and adsorptive functions
Day 3
67% pass the 8-cell stage
5% pyknotic nuclei
B
Organelle redistribution
Disrupted microfilaments
Disrupted microtubules
Mitochondrial swelling
Disrupted secretory and adsorptive functions
Day 3
25 % pass the 8-cell stage)
22% pyknotic nuclei

heat shock in mice. For example, Ozawa et al. (2002) showed an increase in the amount of hydrogen peroxide in two-cell mouse embryos after heat sbock. However, effects of heat shock on development of the bovine-two cell embryo appears not to involve an increase in reactive oxygen species. Experiments were not conducted to monitor free radical production. However, it was bypothesized that if heat shock increases free radical production, it would decrease intracellular content of GSH, one of the major cytosolic antioxidants. There was, however, no reduction in GSH content caused by heat shock (Chapter 4). Furthermore, effects of heat shock on embryonic development were not increased in cultures in atmospheric oxygen as compared to embryos cultured in 5% oxygen (Chapters 2 and 4). As previously stated the mouse and the bovine embryo turn on their genome at different times during development. The first burst of transcription occurs in the mouse embryos around the two-cell stage (Schultz, 1993) while in the bovine embryo genome activation does not occur until the eight-cell stage to 16-cell stage (Memili and First, 2000) . This difference between embryos of both species could explain wby heat shock studies using bovine embryos have such poor results with antioxidants. However, these conclusions still need confirmation by measuring free radicals directly by fluorescent dyes or spin-trap techniques. An attempt was made to detect free radical production in two-cell bovine embryos as a result of heat shock but the lack of sensitivity of the machine made it impossible to do so. Perhaps in the future when more sensitive equipment are made available such experiments will be successful.

The observation that overall morphology and response to heat shock was similar between embryos produced in vivo and in vitro implies that the latter can be a good model for understanding embryonic responses to heat shock. This makes efforts to

understand the pathologies caused by stresses such as heat shock much more feasible as in vitro embryos are easy to obtain, to manipulate, and to produce in high numbers to allow for various measurements. Once pathologies are understood attempts can be made to incorporate the knowledge gathered in in vitro-produced embryos to improve the in vivo-produced embryo while in its normal environment.

To sum up, work outlined in this dissertation gives evidence that the two-cell embryo is very susceptible to the effects of a mild elevation in temperature. The blastomeres at this early stage are not equipped to allow for a complete recovery from the cytoskeletal rearrangement, mitochondrial swelling, and/or other cellular changes induced by heat shock so that a catastrophic outcome is the common fate of the heat-shocked embryo. Perhaps the knowledge gathered in this work will allow for future experimental approaches to reduce the deleterious effects of heat shock on embryos and thereby alleviate infertility in cattle caused by heat stress.

APPENDIX
PHOTO GALLERY OF TWO-CELL BOVINE EMBRYOS

An abundance of interesting, unusual, and artistically-appealing images were generated as part of the work reported in this thesis. A few of these images are placed in this appendix.

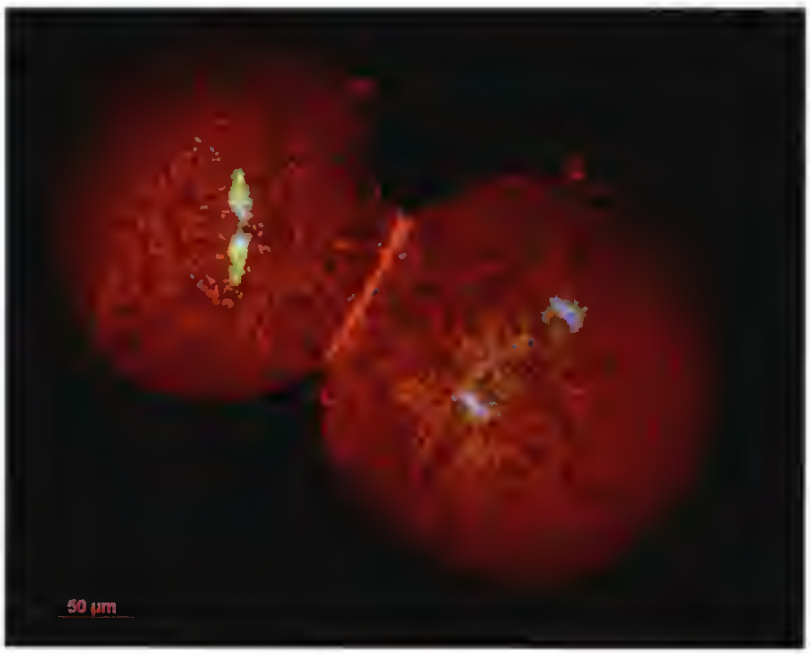


Figure A-1. Fluorescent labeling of chromatin, microfilaments, and microtubules in a two-cell bovine embryo. Chromatin was labeled with Hoechst 33342, microfilaments with Alexa Fluor 594 phalloidin (red) and microtubules with anti-bovine-antitubulin labeled with Zenon Alexa Fluor 488 mouse IgG_1 labeling kit (green).

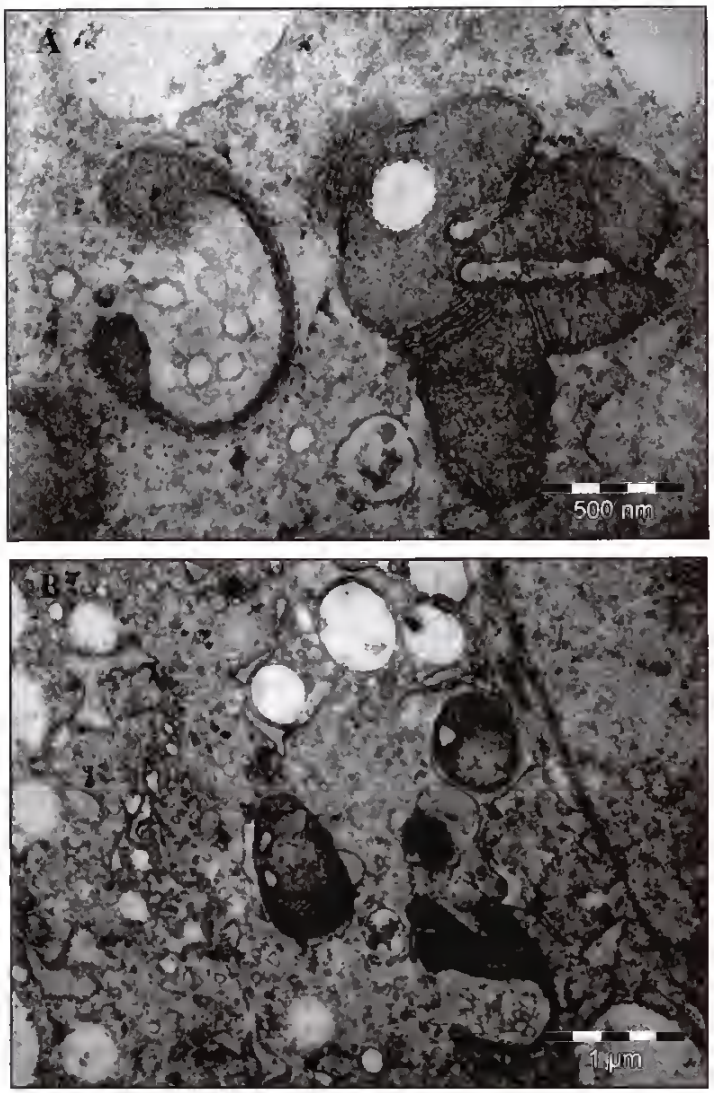


Figure A-2. Unusual mitochondria. Embryos were processed for electron microscopy as explained in chapter 3.

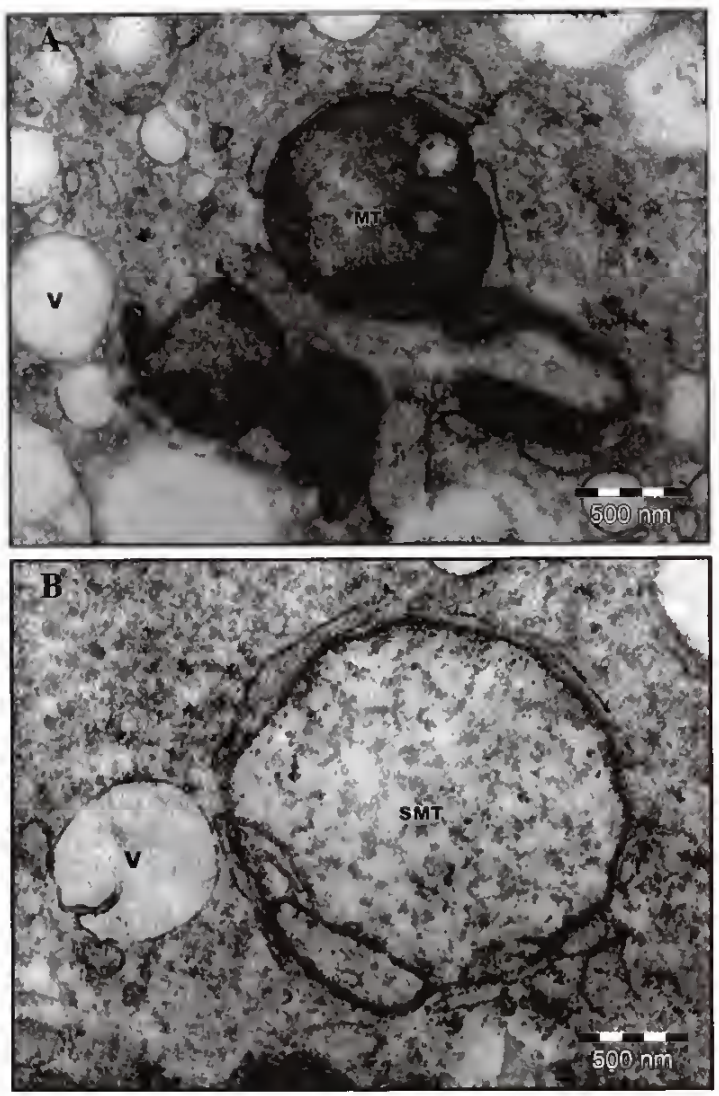


Figure A-3. Comparison of normal (A) and swollen (B) mitochondria in two cell bovine embryos. Embryos were processed for electron microscopy as described in chapter 3.

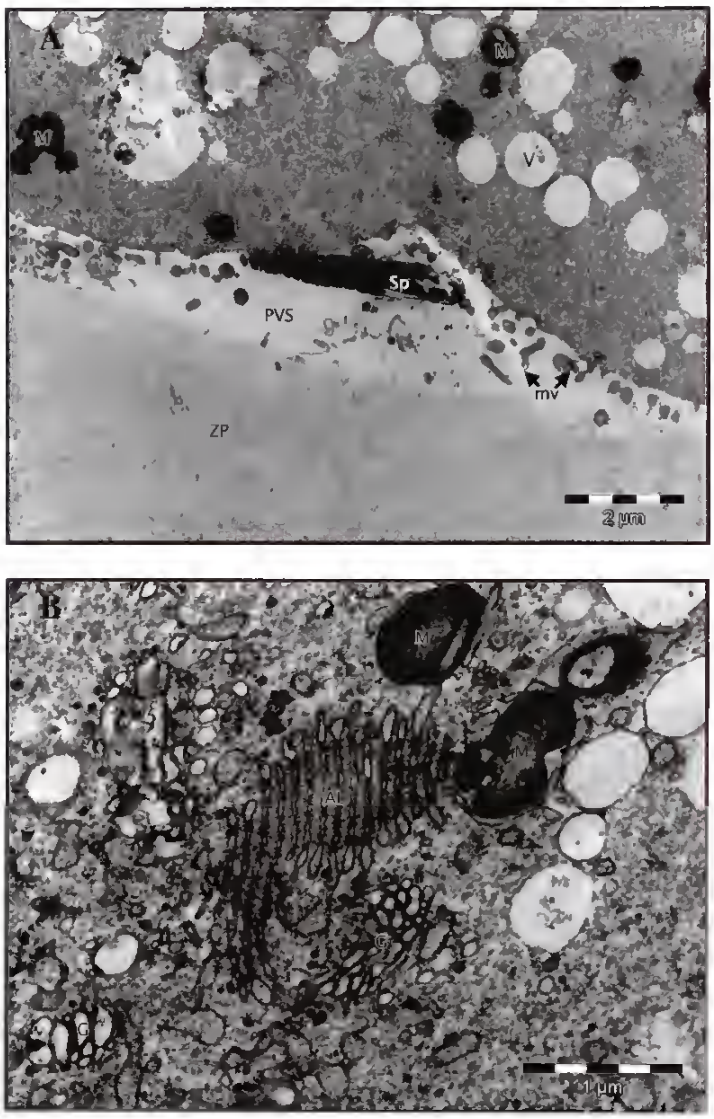
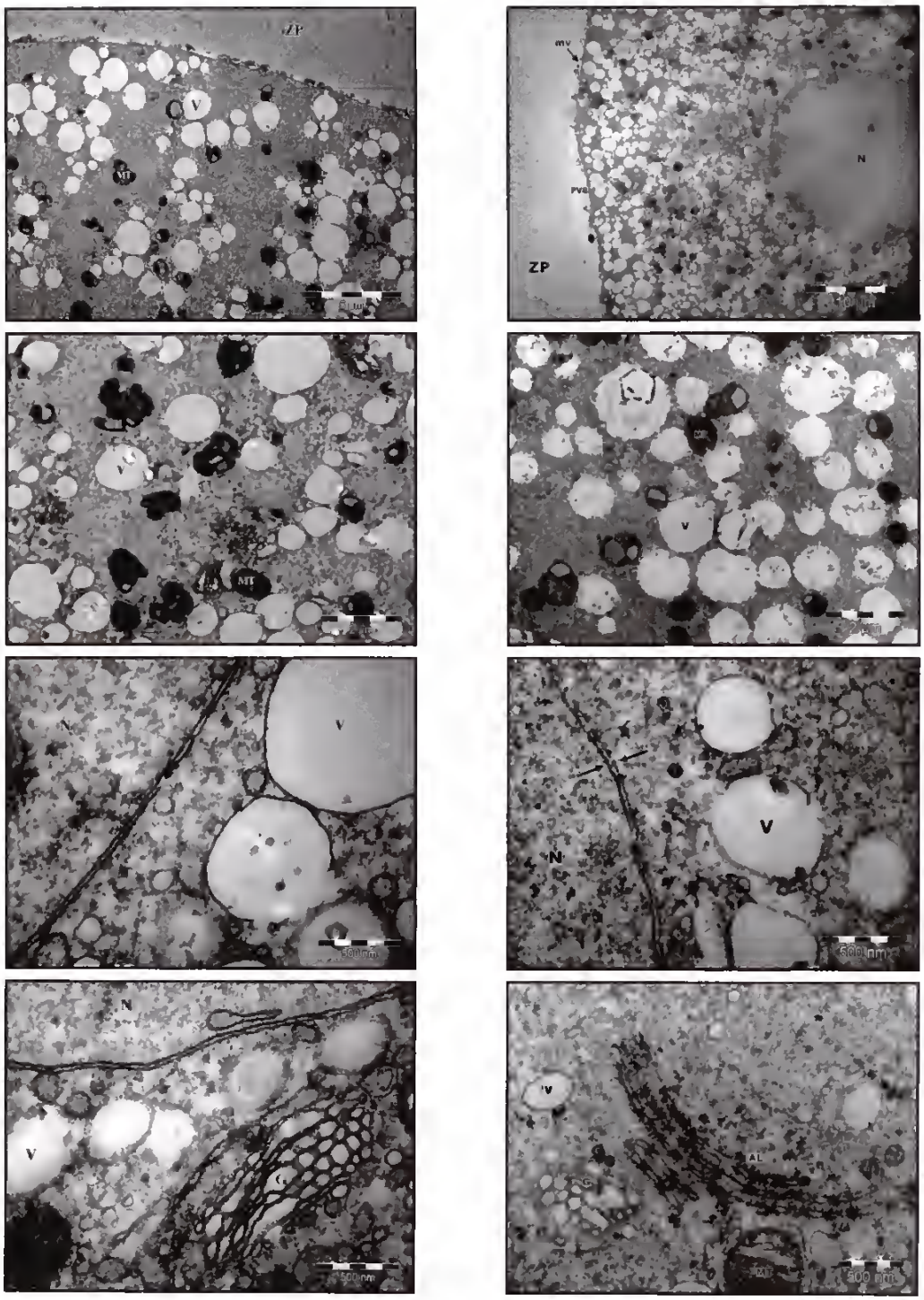


Figure A-4. Assorted electron micrographs of two-cell embryos. An electron micrograph demonstrating a sperm head apposed to the plasma membrane of one of the blastomere of a two-cell bovine embryo is shown in Panel A. Panel B demonstrates cytoplasmic organelles normally found in two-cell embryos. Embryos were processed for electron microscopy as described in chapter 3. Sp = sperm head, AL = annulate lamellae, M = mitochondrion, G = Golgi apparatus, PVS = perivitelline space, ZP = zona pellucida, V= vesicle.

Figure A-5. Electron micrographs comparing the ultrastructural morphology of in vivo and in vitro produced two-cell embryos. Embryos were prepared for ultrastructural examinations as described in chapter 3. AL = annulate lamellae, M = mitochondrion, G = Golgi apparatus, PVS = perivitelline space, ZP = zona pellucida, V= vesicle, mv = microvilli.

In vivo produced

In vitro produced



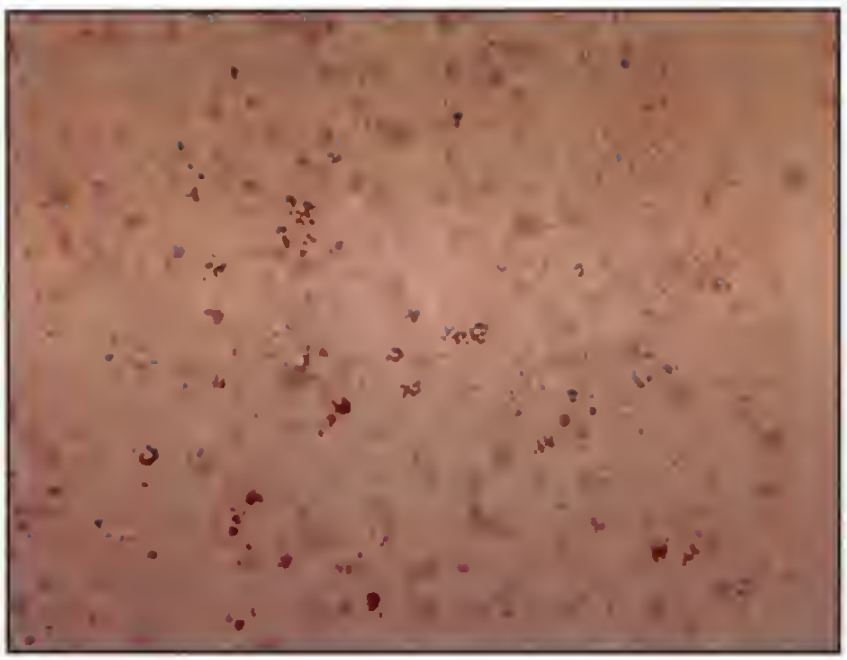


Figure A-6. Periodic acid Schiff assay on two-cell bovine embryos. The procedure for localization of carbohydrate-containing molecules was as previously described (Erdos, 1986). Briefly, embryos were fixed, embedded, and sectioned as described in chapter 3. Semi-thin sections (500 nm) were oxidized with 1% periodic acid at room temperature. Sections were rinsed in distilled water prior to an incubation at 60.0°C with silver methenamine (2 ml 5% $AgNO_3$ + 18 ml of 3% (w/v) methenamine + 2 ml 2% (w/v) sodium borate – pH 8-9). Sections were thoroughly rinsed with distilled water and incubated in a 5% solution of sodium thiosulfate prior to a final wash with distilled water. Note that the positive reaction (brown precipitate) is localized very specifically within cytoplasmic vesicles. Sections were counterstained with Toluidine blue O stain as described in chapter 3.

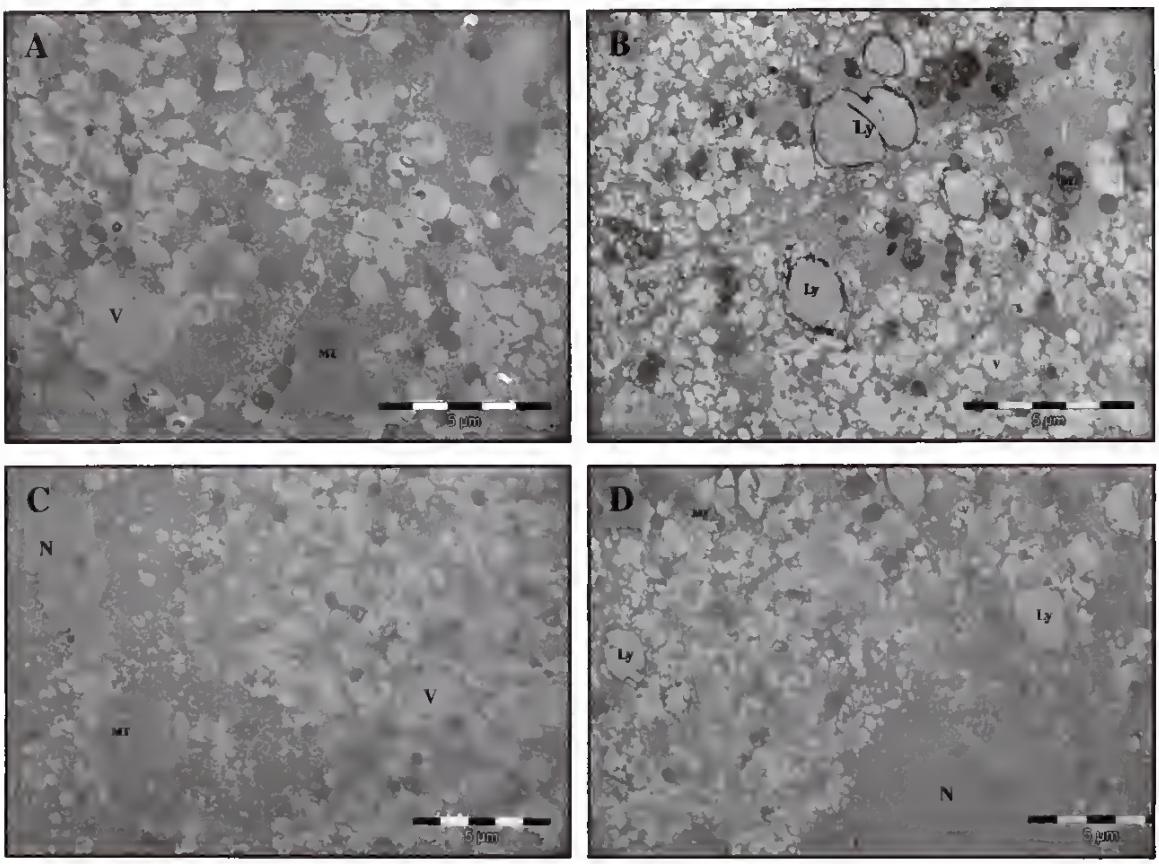


Figure A-7. Transmission electron micrographs demonstrating localization of aryl sulfatase in two-cell bovine embryos. The lysosomal marker enzyme aryl sulfatase was localized as described previously (Van Hekken and Eigel, 1990). Briefly, embryos were fixed in 3% (v/v) glutaraldehyde - 1% (v/v) paraformaldehyde in .067 M sodium cacodylate buffer (pH 7.2). Embryos were then incubated with a reaction mixture (3 ml 0.1 M acetate buffer, pH 5.5 + 0.6% (w/v) p-nitrocatechol sulfate + 1 ml 24% (w/v) $Pb(NO_3)_2$; Panels B and D) or a control mixture (0.1 M acetate buffer; Panels A and C) at 37.0°C. Embryos were then washed in cold acetate buffer prior to dehydration, embedding and section as described in chapter 3 except that ultra-thin sections were NOT post-stained (hence the lack of contrast).

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BIOGRAPHICAL SKETCH

Rocío Melissa Rivera Varas was born on July 30, 1968, in San Juan, Puerto Rico. She is the third child of José D. Rivera Ortíz and Zaida Varas Muñoz. She went to high school at Colegio Nuestra Señora de la Merced in Hato Rey, Puerto Rico. After graduating from high school in 1986, Rivera went to college at the University of Puerto Rico, Mayagüez campus, where she studied for two years before transferring to Iowa State University in Ames, Iowa. There, she completed two Bachelors of Science programs, one in Animal Science and one in Dairy Science, in May of 1993. During her undergraduate work, Rivera worked as a laboratory assistant in the laboratory of Dr. Steve Ford. Following completion of her undergraduate studies, she earned a Master of Science degree in reproductive biology in December, 1995 while working under Dr. Ford's direction. The title of her master's thesis was "A Comparison of Preimplantation Development of Embryos from Chinese Meishan and Yorkshire Pig Breeds". After finishing the master's degree she moved to Gainesville, Florida, to work as a laboratory technician in the laboratory of Dr. Peter J. Hansen at the University of Florida in Gainesville, a job she held from May, 1996 until September 15, 2003. In 1998, she entered the Ph.D. program in the Animal Molecular and Cell Biology Program at the University of Florida under the supervision of Dr. Peter J. Hansen. Upon completion of the requirements of the Doctor of Philosophy degree, she will move to Philadelphia, Pennsylvania, to begin post-doctoral training in the area of genomic imprinting in the laboratory of Dr. Richard Schultz at the University of Pennsylvania.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Animal Sciences

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This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

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